BIOCHEMICAL INVESTIGATIONS INTO PROBLEMS RELATING TO EPILEPSY

by

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3. Moir, A. T. B., Halliday, J. and Williams, I. R. (1971) Lack of effect of folic-acid administration on cerebral metabolism. Lancet, p.798.

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GENERAL SUMMARY

Investigations have been carried out in three separate areas of research related to epilepsy.

I. In the first of these investigations an attempt was made to clarify a possible role of folic acid derivatives in epilepsy. This investigation took the form of two self-contained studies involving dogs.

A. The first study concerned the effects of anticonvulsant treatment on

(a) folate activity in cerebrospinal fluid (c.s.f.).

(b) the concentrations of 5-hydroxyindol-3-ylacetic acid (5-HIAA) and homovanillic acid (HVA) in c.s.f.

Routine sampling of ventricular and cisternal c.s.f. in the dog was achieved by the implantation of permanent guide tubes to the lateral ventricle and the cisterna magna. 5-HIAA and HVA in c.s.f. samples were estimated by fluorescence assay techniques. Folate activity in c.s.f. and plasma samples was estimated microbiologically with a folate-dependent strain of l.casei.

In control studies the c.s.f. folate activity was found to be between 2 and 7 times higher than the plasma folate activity. A positive correlation was found between c.s.f. and plasma folate activities. HVA and 5-HIAA studies confirmed the concentration gradient which exists for these two

acids between the lateral ventricular c.s.f. and the cisternal c.s.f.

Selected anticonvulsant drugs (diphenylhydantoin, 7.5 mg/kg; phenobarbitone, 15 mg/kg; sulthiame 15 mg/kg; carbamazepine 15 mg/kg) were administered daily to the dogs for a period of five weeks. During this period no change was seen in the folate activity of either c.s.f. or plasma. The anticonvulsants were also without effect on the concentrations of HVA and 5-HIAA in c.s.f.

These findings are discussed in relation to the folate deficiency states produced in man by chronic anticonvulsant therapy.

B. The second study in the folate investigations was concerned with the effect of oral folic acid administration ~~or~~ on

(a) folate activity in plasma, plasma ultrafiltrate and c.s.f.

(b) the concentrations of HVA and 5-HIAA in the c.s.f.

During the control period of this study no correlation was found between c.s.f. and plasma folate activity, such as was found in the first study. Possible reasons to account for this discrepancy are discussed. Estimation of folate activity in plasma and plasma ultrafiltrate confirmed that much of the folate activity in dog plasma is protein-bound.

Folic acid (31 mg/kg) was administered daily to dogs over a 5-week period. During this treatment period the mean plasma folate activity was 10 times higher than normal; plasma ultrafiltrate folate activity was 5 times higher the normal; but c.s.f. folate activity was unchanged. These findings were interpreted as evidence for an increased plasma level of a folate derivative, probably folic acid itself, which was more strongly bound to protein than normal plasma folate derivatives, and was unable to enter the c.s.f. In two experiments in the rabbit no evidence was found for the active transport of methyl-tetrahydrofolate (normally the predominant form of folate in the plasma) into the c.s.f. HVA and 5-HIAA concentrations in the c.s.f. were unaltered during the period of folic acid treatment. It was concluded that oral folic acid administration is unlikely to have any significant effect on normal cerebral function.

The implications of this study are discussed in relation to

(a) the role of folic acid derivatives in epilepsy and mental illness.

(b) the mechanisms responsible for the normal distribution of folate activity between c.s.f. and plasma.

II. The second investigation concerned the effects of barbiturate anaesthetics and anticonvulsant drugs on the c.s.f. potassium fluxes of the conscious dog.

A new technique was developed for "open" perfusion of the cerebroventricular system of the conscious dog. The dog was perfused from lateral ventricle to cisterna magna with artificial c.s.f. containing inulin, and tracer amounts of ^{42}K. ^{42}K, total potassium and inulin assays were performed on the inflow and outflow fluids. From this data it was possible to calculate, for each sample of outflow fluid collected, values for both the potassium efflux from, and the potassium influx into, the c.s.f. After a period of between 65-100 minutes of perfusion a "steady-state" was reached in which the potassium fluxes, as measured by this technique, were relatively constant. Drugs actions were studied by administering the drug during the "steady-state" period and ascertaining the effect upon the "steady-state" potassium fluxes.

It was shown that -

(1) The barbiturate anaesthetics, sodium pentobarbitone and sodium thiopentone, in doses sufficient to induce light anaesthesia, may depress both potassium influx into, and potassium efflux from c.s.f. Both fluxes were affected equally on any one occasion. Flux alterations of up to 50% were observed.

(2) Diazepam has a consistent depressant effect on the potassium fluxes of the c.s.f. This effect was less marked, but more consistently observed, than in the case of the barbiturate anaesthetics.

(3) Diphenylhydantoin has no significant effect on potassium efflux from the c.s.f. but may have a slight depressant effect on potassium influx into the c.s.f.

(4) Paraldehyde in a dose sufficient to induce light anaesthesia slightly increases the c.s.f. potassium fluxes.

These results are discussed in relation to

(a) previously published studies on c.s.f. potassium fluxes in anaesthetised animals

(b) possible mechanisms of action of these drugs.

The effects of the barbiturate anaesthetics and diazepam were interpreted as being secondary to a decrease in potassium exchange between brain intracellular and extracellular compartments.

It was concluded that the anaesthetic or anticonvulsant action of a drug is not related to the drug's effect on c.s.f. potassium fluxes.

III. The third investigation concerned the development and preliminary application of a method for the analysis of GABA and associated amino acids in small areas of tissue from the primary and secondary epileptic foci of rats with a cobalt-induced epileptogenic lesion.

The existing specific enzymic methods for GABA analysis were examined and found to be either too insensitive or too complex to act as suitable micro-methods. The standard techniques for amino acid analysis, involving column separation and colorimetric estimation were also considered to be too insensitive.

Reaction of the amino group of amino acids with 1-dimethyl-amino-napthalene-5-sulphonyl chloride (dansyl chloride) produces stable, highly-fluorescent derivatives which can be readily separated by thin-layer chromatography.

A highly sensitive isotope dilution assay, based on the preparation of dansyl derivatives, was evolved for the estimation of free amino acids in brain tissue.

The tissue was homogenised in perchloric acid and known amounts of ^{14}C-amino acid standards were added to an aliquot of the perchlorate extract to act as internal standards for recovery of amino acid through the method. The extract was then taken to pH 9.3 - 9.8 with potassium carbonate solution, precipitating perchlorate as its insoluble potassium salt. An aliquot of the alkaline extract was dansylated with an equal volume of H^3-dansyl chloride in acetone. After a 30 minute reaction period the dansylation mixture was taken to dryness and extracted with acetone: glacial acetic acid solution (3:2). An aliquot of this extract was applied to a polyamide micro-chromatography plate which was then developed in two dimensions. Under u.v. light specific dansyl-amino acid spots were identified by their characteristic position on the developed plate. These spots were cut from the plate and counted for ^3H and ^{14}C activity in a liquid scintillation counter.

The total amount of dansyl-amino acid in a spot was calculated from its ^3H activity. Combining this figure with the ^{14}C activity

of the spot allowed calculation of the specific activity (w.r.t. ^{14}C) of the dansyl-amino acid in the spot. Comparison of this specific activity with the specific activity of the ^{14}C-amino acid standard which had been added as a recovery standard gave a measure of the dilution of exogenous radioactive amino acid by endogenous non-radioactive amino acid. From this dilution factor it was possible to calculate the concentration of endogenous amino acid in the original perchlorate extract of brain tissue. Determination of the weight of protein precipitated from the tissue sample by the perchloric acid extraction allowed calculation of the amino acid content of tissue in terms of μmoles amino acid per 100 mg protein.

The two dimensional chromatography did not achieve a separation of dansyl-glutamine and dansyl-threonine; as a result glutamine and threonine were estimated as the sum total of their two concentrations.

The method was applied to the estimation of control values for GABA, glutamate, glutamine/threonine, glycine, aspartate and hydroxyproline levels in rat cortex. The values obtained for GABA, glutamate, glutamine/threonine and glycine were highly reproducible and in good agreement with previously published values. Aspartate was not satisfactorily estimated. Hydroxyproline was not detected in rat cortex.

The dansylation method was applied to the analysis of brain tissue from three rats with 11 day-old cobalt-induced epileptogenic

lesions in the frontal cortex. There was evidence that GABA and glutamate levels were lowered, and glutamine/threonine and glycine levels raised, in the region of the secondary epileptogenic ("mirror") focus. This was the site from which epileptiform spike discharges were most frequent.

The significance of these findings have been discussed along with general comments on the problems of amino acid studies in brain. It was concluded that disorders in amino acid metabolism may be involved in epileptic processes.

GENERAL INTRODUCTION

Epilepsy has long been, and continues to be, one of the most challenging problems in the field of medicine. Man's efforts to cure this affliction may indeed date back to the stone age, if, as has been conjectured, evidence of successful trepanning in skulls from the Neolithic period indicate early attempts at a remedy. The first scientific work on epilepsy was a treatise "On the Sacred Disease" written in Greece in 450 B.C. This early text, which has been attributed to Hippocrates, criticised the belief, then popularly held, that epilepsy was a supernatural possession of the body by devils and proposed that it was, rather, a hereditary disease of the brain. A distinction was also drawn between epilepsy occurring in the absence of any particular aetiological factors, sometimes referred to as idiopathic epilepsy, and symptomatic epilepsy occurring in the presence of an obvious cause such as a head injury or a fever. Here also we find the first mention of the aura which often precedes a seizure, and of the influence of such factors as age and temperament upon the nature of the epilepsy.

In the succeeding two thousand years little was added to the Hippocratic concepts of epilepsy and indeed, not until the work of John Hughlings Jackson in the nineteenth century do we find a satisfactory physiological explanation of the disease. After careful clinical examination of certain localised types

of seizure (now named Jacksonian), Jackson proposed that epilepsy resulted from occasional, sudden, excessive, rapid and local discharges of the grey matter of the brain. With the later introduction of techniques to measure the electrical activity of the brain (electroencephalography and depth electrode recording) this hypothesis has been fully confirmed.

A modern concept of epilepsy, or more correctly the epilepsies, is of a symptom complex characterised by recurrent, paroxysmal aberrations of brain function, usually brief and self-limited. These aberrations in function result from abnormal neuronal activity of the type proposed by Jackson, but the exact physiochemical mechanisms involved in the production of the epileptic neuronal activity are not known. It is assumed that the basic fault is some structural or functional pathological process in the brain. In many cases one can demonstrate a discrete epileptic focus with an organic lesion, as in epilepsy following haemorrhagic or ischaemic damage to the brain tissue, or in epilepsy due to a brain tumour. In other cases no obvious organic lesion can be demonstrated (cryptogenic or idiopathic epilepsy) but even in many of these cases an organic defect may be suggested by inheritance of the disease.

From an epileptic focus of electrically unstable neurones the epileptic discharge can spread to normal neural tissue producing synchronous, paroxysmal discharges of large groups of neurones. When this happens a clinical seizure ensues. The

type of clinical seizure pattern which is observed depends little on the original cause of the epileptic focus but rather on the anatomical location of the focus and on its pattern of recruitment of normal neurones.

Apart from the mechanisms responsible for the generation of an epileptic focus there are a number of general physiological factors which affect the type and frequency of clinical seizure produced. These factors may be considered in terms of their producing an increase or decrease in seizure threshold.

Genetic predisposition is an obvious factor in many types of human epilepsy. In animals it has been possible by selective breeding to produce certain strains which have a much reduced seizure threshold to stimuli such as flickering lights or loud noises. Age and cerebral maturation are also important factors. For instance febrile convulsions occur almost entirely in children below the age of five. The occurrence of petit mal is also linked to age, most cases occurring between the ages of four and eight. In some laboratory animals electroshock seizure threshold is high at birth and decreases as the brain matures. The decrease in electroshock threshold correlates well with an increase in carbonic anhydrase activity in the brain but this is only one of many changes occurring during cerebral maturation.

Seizure threshold is also highly dependent on the energy supply to the brain. If this is reduced, as in hypoglycaemia, there is a sharp decrease in seizure threshold. Hypoxia has a

similar effect. Severe anoxia on the other hand increases seizure threshold, presumably by limiting any kind of neuronal activity.

A drop in CO_2 tension in the blood reduces seizure threshold (hyperventilation is a recognised way of stimulating seizures in epileptic patients) while an increase in CO_2 tension increases seizure threshold. This latter action may be responsible for the anticonvulsant effect of carbonic anhydrase inhibitors.

Diet can also influence epilepsy and it has been found that a ketogenic diet (high in fat, low in carbohydrate) increases seizure threshold. Disorders in water and electrolyte balance produce alterations in seizure threshold, both water intoxication and hyponatraemia tending to induce convulsions, and water deprivation tending to depress them. Hormonal factors can also influence brain excitability. Adrenal cortical insufficiency decreases, while deoxycorticosterone increases seizure threshold. Corticotrophin also increases seizure threshold and has been successfully used to alleviate some types of epilepsy. Thyroid hormones increase brain excitability firstly by a direct stimulant effect on the central nervous system and secondly by an indirect effect on the adrenal cortex. Many of these changes in seizure threshold produced by hormones may be secondary to changes in electrolyte and water balance.

From this short introduction it is obvious that many different pathways and systems are involved in epilepsy and in this thesis we have attempted to isolate and study three

different mechanisms which have been implicated in the aetiology or control of this common disease. The three subjects studied were the role of folic acid derivatives in epilepsy; the effect of anticonvulsant drugs on potassium transport in the brain; and amino acid changes in a model epileptogenic focus.

SECTION I

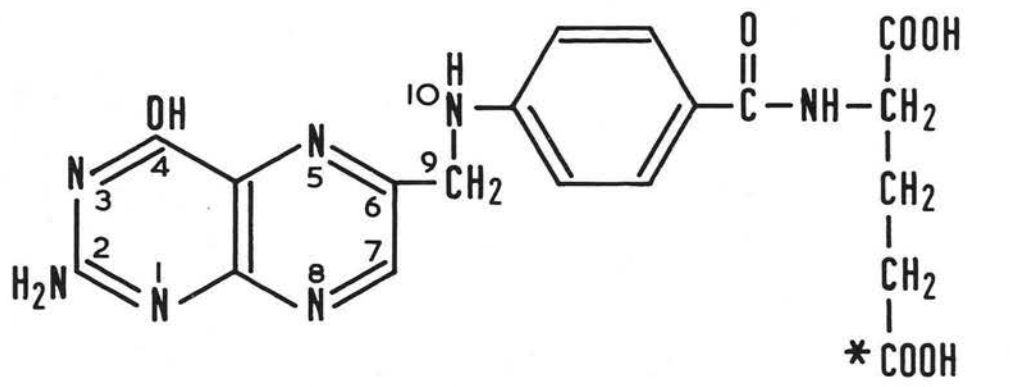
FOLIC ACID METABOLISM AND ANTICONVULSANTS

INTRODUCTION

The Identification and Estimation of "Folate"

Since the first recognition in the 1930's of an unidentified growth factor which was required by animals (Wills and Stewart, 1935; Day, Langston and Darby, 1938; Hogan and Parrot, 1939; Stokstad and Manning 1938) and certain microorganisms (Snell and Peterson, 1940; Mitchell, Snell and Williams, 1941) there has been an ever increasing awareness of the variety and complexity of the involvement of the folic acid coenzymes in general metabolism. The present state of this field of study has been splendidly reviewed by Stokstad and Koch (1967) and Chanarin (1969), and it is also the subject of a very comprehensive monograph by Blakely (1969). This introduction is only a brief sketch of the relevant background and for more detailed information the reader is directed to these excellent sources.

The parent substance, folic acid (Fig. 1:1), is itself not biologically active and it is the dihydro or tetrahydro derivatives which actually function as coenzymes in a wide range of reactions, principally in the field of one-carbon transfer metabolism. Fig. 1:2 illustrates the various forms of folate which are known to occur in biological material and indicates some of the relationships between them. Fig. 1:2 also lists some of the compounds which provide one-carbon fragments for the folate coenzymes and some of the compounds in whose synthesis



FOLIC ACID

* Carbon atom at which additiond glutamyl residues are linked. → folate polyglutamate conjugates.

FIG. 1:1 The structure of folic acid

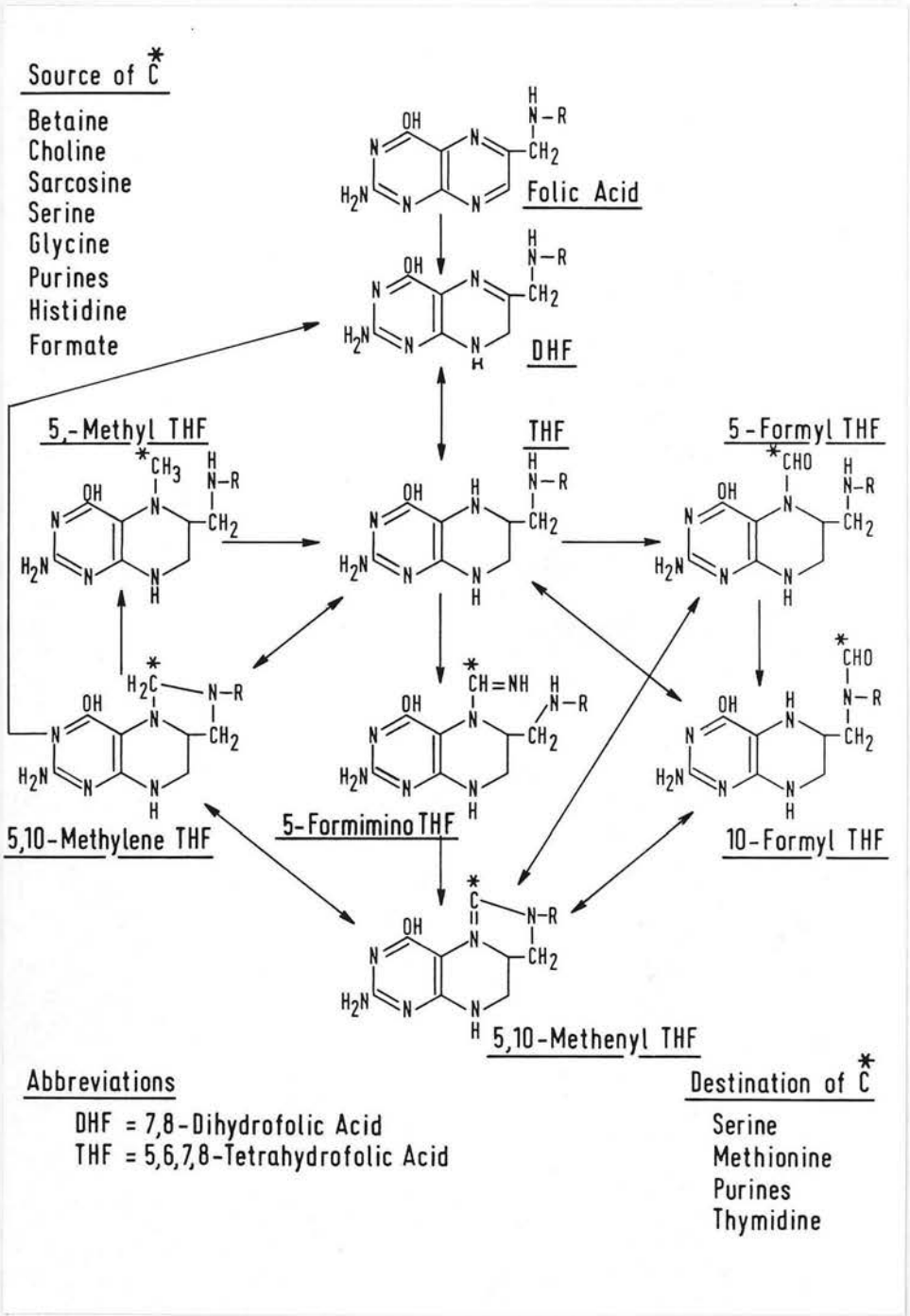


FIG. 1:2 Metabolic interconversions of folic acid derivatives

one-carbon units from folate coenzymes have played an essential part. The picture presented by Fig. 1:2, complex though it is, is further complicated by the fact that most of the folate in biological samples is not present in a simple form, but is linked, through peptide links at the γ position of the glutamate moiety, to further glutamate residues. Such polyglutamate conjugates make up the bulk of folate derivatives in the liver (Noronha and Silverman, 1962; Bird, McGlohon and Vaitkus, 1965) and the red cell (Noronha and Aboobaker, 1963). The work of Wright (1958) demonstrated that in some 'in vitro' situations these conjugates are active while the simple derivatives are inactive.

It is possible therefore, that in one biological sample we might encounter folate derivatives differing from each other in three separate ways. Firstly they might differ in the number of covalently linked γ glutamyl residues, secondly in the nature of the substituents, if any, on the nitrogen atoms at positions 5 and 10, and finally in the state of oxidation of the pteridine nucleus. A further complication lies in the fact that most of the tetrahydro derivatives of folic acid are highly labile and when exposed to air they may be oxidised to a relatively stable form such as N5-formyltetrahydrofolic acid, or be oxidatively cleaved to form a pterin and para-aminobenzoylglutamic acid. In order to prevent such interconversion or destruction it is necessary to protect the sample with a reducing agent such

as ascorbate (Noronha and Silverman, 1962; Bird et al., 1965) or mercaptoethanol (Copenhaver and O'Brien, 1969). Once the labile folate derivatives in a sample have been protected from oxidation there still remains the critical problem of how to assay them. Although folic acid and its derivatives can be estimated colourimetrically after a diazotisation procedure (Hutchings, Stokstad, Boothe, Mowat, Waller, Angier, Semb and Subbarow, 1948; Glazko and Wolf, 1949) this method is not sensitive enough to assay the nanogram amounts of folate found in a millilitre of c.s.f. or plasma, nor does it give any indication which derivatives are present. Allfrey, Tepley, Geffen and King (1949) reported a fluorimetric method for the assay of folic acid in plant and animal tissues but it also is lacking in sensitivity and specificity. More recently Netrawali, Radhakrishnamurty and Sreenivasan (1964) were successful in using a fluorimetric method to measure the endogenous levels of 5-formyl tetrahydrofolate in tissue. The studies of Uyeda and Rabinowitz (1963) would certainly suggest that once extraction and separation techniques have been improved there would be considerable potential in final fluorescence assay procedures. Udenfriend (1969) has recently reviewed this subject.

At the present moment however the only assay technique with the necessary sensitivity and specificity is microbiological assay. Table 1.1 shows the various test micro-organisms which have been used to assay folate and their specificities in terms

TABLE 1.1

COMPOUND	Growth Response		
	L. caseii	S. faecalis	P. cerevisiae
folic acid	+	+	-
dihydrofolic acid (DHF)	+	+	-
tetrahydrofolic acid (THF)	+	+	+
5-formylTHF	+	+	+
10-formylTHF	+	+	+
5-methylTHF	+	-	-
folylglutamate	+	+	-
folyldiglutamate	+	-	-
folylhexaglutamate	-	-	-

\+ = a response at least 50% of the maximum

\- = a response less than 5% of the maximum.

of sensitivity to the different forms of folate. Unfortunately the different growth responses of these bacteria to different folate derivatives are not of an "all or none" nature and therefore cannot be used for the absolute determination of one derivative in the presence of one or more of the others. Nevertheless differential assay before and after treatment of the sample with a 'conjugase' enzyme preparation is a technique of considerable value; and it was by this means that Herbert, Larrabee and Buchanan (1962) were able to show that most of the folate

activity in the serum was in the form of the methyltetrahydrofolate derivative. The 'conjugase' treatment consists of treating the sample with an enzyme preparation containing γ glutamyl carboxypeptidase, an enzyme which is capable of splitting off glutamate residues from folylpolyglutamate conjugates to give either simple folates or at least lower order polyglutamates. This is a necessary procedure prior to total folate assay since, as Table 1.1 indicates, the three commonly employed test organisms are incapable of utilising high order folylpolyglutamates. Wolff, Drouet and Karlin, (1949) demonstrated that plasma itself contained such a peptidase and it has been found in many other animal tissues, invertebrates, plants and bacteria. A detailed review of the peptidases occurrence and characteristics is included in the relevant chapter of Blakely (1969).

Therefore it can be seen that to estimate the total folate content of a sample with such an organism as Lactobacillus casei (L. casei), it is first necessary to ensure that any derivatives with more than two extra glutamate residues are degraded by 'conjugase' treatment to simpler forms (see Table 1.1). Further sophistication to examine the individual derivatives separately is possible by two means. Firstly, as was mentioned earlier, the sample may be analysed before and after 'conjugase' treatment, with the three different bacterial strains, L. casei, s.faecalis and p.cerevisiae. On three counts however this

technique is far from perfect. Firstly it gives us no information on the amounts of polyglutamate fractions of various chain lengths. Secondly the differential responses are far from absolute; and for some forms, e.g. formyltetrahydrofolate, there is no differentiation at all. Thirdly there has been a suspicion that some of the conjugase preparations themselves contain contaminating folate.

The second way in which some sophistication can be brought into an examination of tissue folates is to carry out some sort of preliminary extraction and separation procedure. The production of a non-proteinaceous aqueous extract (usually by heating, followed by centrifugation) with the subsequent use of paper chromatography (Winsten and Eigen, 1950), or electrophoresis (Hillcoat and Blakely, 1964), or column chromatography with a substituted cellulose (Silverman, Law and Kaufman, 1961; Noronha and Silverman, 1962) has been the standard procedure. Once the various derivatives have been separated in this way they have been assayed microbiologically. These techniques however suffer from the disadvantage that they require relatively large amounts (milligrams) of folate to be present in the tissue samples being analysed. Copenhaver and O'Brien (1969) attempted to break away from the reliance on biological assay, using an ion exchange resin thin layer chromatographic technique followed by fluorimetric and colourimetric estimation procedures.

The final estimations however require microgram amounts of each derivative and it is therefore not readily applicable to assay of small amounts of folate, though as the authors point out it may be of considerable use in experiments involving radioactive tracer folates.

It was against this background of complex and unsatisfactory assay techniques that we set out to examine in a fairly simple manner some aspects of folate metabolism, particularly the effects of anticonvulsant and folic acid therapies.

SECTION 1A: The Effect of Selected Anticonvulsants on (a) the Concentration of Folate in Cerebrospinal Fluid and Plasma and (b) the Concentration of Homovanillic Acid and 5-Hydroxyindol-3-ylacetic Acid in Cerebrospinal Fluid.

Introduction

The first indication of a link between anticonvulsant therapy and folic acid metabolism came in 1952 with a report by Mannheimer, Pakesch, Reimer and Vetter (1952) that diphenylhydantoin might be the agent responsible for a case of megaloblastic anaemia. Subsequent reports confirmed diphenylhydantoin therapy as the probable causal factor in certain other cases of anaemia (Hawkins and Meynell, 1954; Webster, 1954) and indicated that both primidone (Girdwood, 1956; Fuld and Moorhouse, 1956) and phenobarbitone therapy (Hawkins and Meynell, 1956; Hobson,

Selwyn and Mollin, 1956) could result in similar complications.

From these early reports a characteristic pattern emerged. Serum vitamin B12 was normal in those patients in whom the test was done (Badenoch, 1954; Chalmers and Boheimer, 1954; Rhind and Varadi, 1954; Girdwood and Lenman, 1956) and the absorption of radioactively labelled vitamin B12 also appeared to be normal (Badenoch, 1954; Chalmers and Boheimer, 1954; Hawkins and Meynell 1958). The anaemias were, in the majority of cases refractory to cyanocobalamin (vitamin B12) therapy but responsive to oral folic acid therapy (Girdwood and Lenman, 1956; Hawkins and Meynell, 1958). These findings pointed to a folate deficiency anaemia but surprisingly Girdwood and Lenman (1956) could find no evidence of malabsorption of folate after an oral dose of folic acid. Nor could they detect any abnormality in the clearance of folate from blood of the type normally found in folate deficiency states. They therefore suggested that an effective avitaminosis might be present because of competitive inhibition between the structurally similar anticonvulsants and folate coenzymes. While the exact relationship between anticonvulsants and folate metabolism remained a matter of speculation at this stage, the existence of such a link could no longer be doubted after the report by Hawkins and Meynell (1958). They found that about 40% of their patients on anticonvulsants displayed macrocytic changes in the red blood cells which disappeared after folic acid therapy.

The first direct evidence of an effect on folates came with the development of reliable serum folate assays (Baker, Herbert, Frank, Pasher, Hutner, Wasserman and Sabotka, 1959; Waters and Mollin, 1961), when low serum folates were found to be present in anticonvulsant induced anaemias (Kohn, Mollin and Rosenbach, 1961; Druskin, Wallen and Bonagura, 1962). Subsequent large scale surveys of patients on anticonvulsant therapy (Klipstein, 1964; Reynolds, Milner, Matthews and Chanarin, 1966) revealed sub-normal serum folates and macrocytic or megaloblastic changes in a substantial proportion of these patients. Klipstein found a correlation between macrocytosis and low serum folates in his group. On the basis of the accumulated evidence it was proposed (Reynolds et. al., 1966) that the antifolate effects of the three drugs, diphenylhydantoin, primidone and phenobarbitone might be causally related to their therapeutic anticonvulsant effects, perhaps by reducing protein synthesis in the central nervous system. Certainly such a relationship was in accord with the finding reported by Chanarin, Laidlaw, Loughridge and Mollin, (1960) of a patient with a drug-induced anaemia who rapidly developed 'status epilepticus' on two separate occasions when she was treated with folic acid. Also, a later report indicated an increase in fit frequency and severity in 50% of patients on anticonvulsants who were treated with folic acid (Reynolds, 1967). It should be stated that Hawkins and Meynell (1958) were of the opinion that folic acid had the opposite

effect in their group of patients.

One effect of Reynold's hypothesis was to focus attention on the role of folates in the central nervous system; and in this context Herbert and Zalusky (1961) had reported that the folate activity of the c.s.f. was between one and a half and five times greater than that of plasma. The significance of this observation lay in the fact that for many substances, the concentration in c.s.f. may closely reflect the concentration in the extraneuronal fluid of the brain (Davson, 1967). If this were true for folate it would certainly suggest that folate was particularly important for normal functioning of the central nervous system.

The first study on the effect of anticonvulsant therapy on c.s.f. folate activity, by Wells and Casey (1967), found significantly lowered c.s.f. folates in their group of patients but this finding was not confirmed by the study of Weckman and Lehtovaara (1969). However, independent extensive studies by Reynolds, Preece and Chanarin (1969) again found a convincing depression of c.s.f. folates in a group of patients on anticonvulsant therapy.

The investigations to be reported in this section of the thesis were an attempt to clarify further the relationships between anticonvulsants and folic acid metabolism. Dogs were chosen as the experimental animal since, by use of techniques developed in this department it was possible to carry out routine

sampling of ventricular and cisternal c.s.f. The four different drugs selected for administration, diphenylhydantoin, phenobarbitone, carbamazepine and sulthiame were chosen to represent the most important classes and structures of anticonvulsants.

In this investigation we were also concerned to look at possible mechanisms by which a lowered folate concentration in the brain, or indeed interference with folate metabolism in the brain, might lead to an anticonvulsant effect. There is strong evidence that dopamine, noradrenaline and 5-hydroxytryptamine act as modulators of neuronal action in the central nervous system and it is possible that an alteration in the synthesis of these compounds could affect epileptic activity in the brain. It has been shown that both brain tyrosine hydroxylase and brain tryptophan hydroxylase utilise pteridine co-factors (Nagatsu, Levitt and Udenfriend, 1964; Gal, Armstrong and Ginsberg, 1966). Since these two enzymes are the rate limiting steps on the pathways leading to the formation of dopamine and 5-hydroxytryptamine respectively, it is possible that any decrease in co-factor availability might bring about a reduction in synthesis of these amines. This was particularly relevant to the folate studies since the folates themselves are substituted pteridines; and Kaufman and Levenberg (1959) have demonstrated that, in a very similar hydroxylation system, tetrahydrofolate can substitute for the normal pteridine co-factor, albeit less efficiently.

Another objective of the present work has therefore been to look at the effect of anticonvulsants on the turnover of dopamine and 5-hydroxytryptamine in the brain. The principal metabolites of dopamine and 5-hydroxytryptamine in the brain are respectively, homovanillic acid (HVA) and 5-hydroxyindol-3-ylacetic acid (5-HIAA). Since in the dog neither of these acid metabolites can enter c.s.f. from the blood (Ashcroft, Dow and Moir, 1968; Guldberg and Yates, 1968) it seems likely that the HVA and 5-HIAA found in dog c.s.f. are derived from cerebral metabolism. More directly, in a variety of experimental situations it has been demonstrated that the c.s.f. concentrations of these metabolites may reflect their concentrations in the brain itself (Moir, Ashcroft, Crawford, Eccleston and Guldberg, 1970). However, c.s.f. levels of these metabolites depend not only on their rate of production in the brain, but also on their rate of transfer into the c.s.f., and on their transport from the c.s.f. into the blood.

In the dog there is a gradient in the concentrations of both HVA and 5-HIAA between ventricular and cisternal c.s.f. (Guldberg, Ashcroft and Crawford, 1966). This is caused by the removal of these acids from the c.s.f. by an active transport mechanism located probably in the 4th ventricular choroid plexus (Ashcroft, Dow and Moir, 1968; Pullar, 1971). Any interpretation of c.s.f. data must therefore take account of the existence of this transport mechanism and the possible implications of

changes in its activity. Under conditions where the transport of HVA and 5-HIAA remains unaltered, changes in the concentrations of these metabolites in the c.s.f. may be a simple way of detecting changes in the turnover of dopamine and 5-hydroxy-tryptamine in the brain (Moir et. al., 1970). We have therefore measured these concentrations of HVA and 5-HIAA in ventricular and cisternal c.s.f. of the dogs during the control period and then during the period of administration of anticonvulsants.

ANIMAL METHODOLOGY

The animals used in these studies were pure bred adult Beagle dogs. This breed was found to be ideal from the point of view of both size and temperament.

Operative Technique for the Implantation of Ventricular and Cisternal Guide Tubes.

All necessary instruments, swabs and gowns were prepared and sterilised for the operation which was carried out under fully aseptic conditions. Food was withdrawn from the dog 16 hours before the operation but water was not restricted.

Anaesthesia was induced with an intravenous injection of sodium pentobarbitone (Nembutal, Abbot Laboratories) and the syringe and needle were taped into position to allow maintenance doses when necessary. The dog was then placed on the operating table in a prone position and a cuffed and armoured endotracheal tube was inserted into the dog's trachea to ensure a clear airway

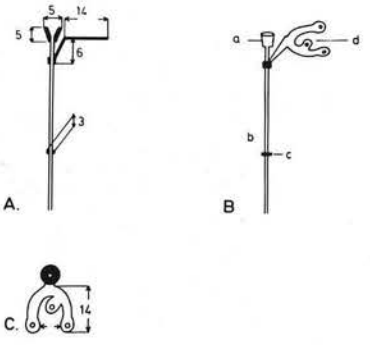


Fig.1

A. CANNULA IN SECTION

B. GENERAL VIEW OF CANNULA

a.) Funnel of cannula
b.) Stainless steel tube
c.) Small collar
d.) Horseshoe shaped plate

C. VIEW OF CANNULA FROM ABOVE (diagramatic)

(Measurements in mm)

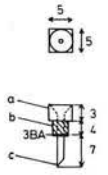


Fig.2

GUIDE TUBE FOR LATERAL VENTRICLE IN DOG BRAIN (Sectional view)

a.) Part situated subcutaneously
b.) Part screwed into skull.
c.) Part inserted into brain, directed towards lateral ventricle.

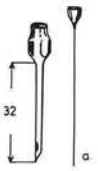


Fig.3

LUER FITTING NEEDLE WITH SIDE HOLE

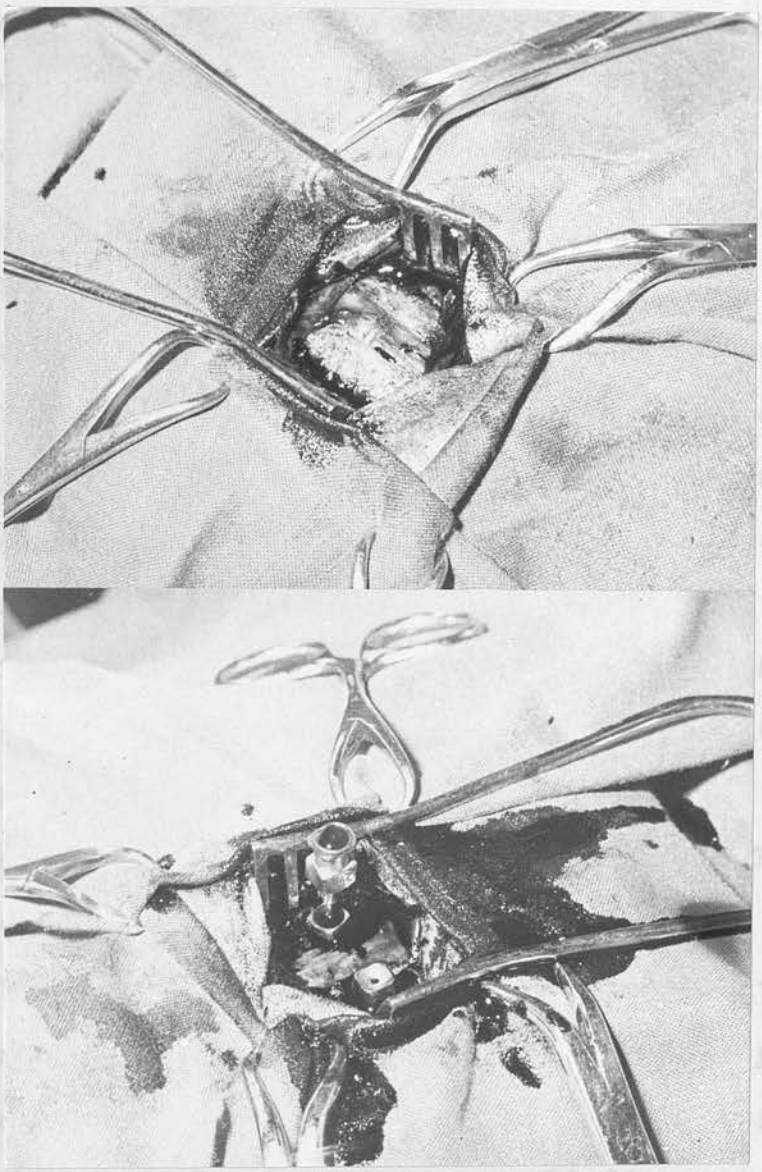
a.) Stainless steel stilette.

FIG. 1:3 Diagram of ventricular and cisternal guide tubes.

throughout the operation. The top of the dog's head and neck was then clipped and shaved and the area was swabbed, first with a 1% solution of cetavlon then with a dilute tincture of iodine. The assistant/anaesthetist then supported the dog's head, keeping the vault of the skull horizontal, and the operating area was delineated with four sterile towels.

The first skin incision was made along the midline from a point 6 cm anterior to the external occipital protuberance to within 2 cm of the protuberance. The edges of the incision were retracted laterally and the temporal muscle incised over the sagittal crest. With a periosteal elevator the muscle and connective tissue were worked away from the midline to expose the vault of the skull (Fig. 1:4). Two scalpel nicks were then made in the bone at points 8 mm out from the midline and 37 mm anterior to the external occipital protruberance. At each point in turn the bone was carefully drilled through with a hand drill (3 mm diam.), the hole tapped with a 3Ba tap, and the ventricular guide tube (Fig. 1:3) screwed firmly into place with a box spanner. To verify a successful placement a 21 gauge sterile needle was passed slowly down the guide tube until cerebrospinal fluid (c.s.f.) welled up into the cup of the needle (Fig. 1:4). This phase of the operation was completed by closing the sub-cutaneous fascia over the guide tubes with continuous interlocking suture of 3.0 chromic gut, and stitching up the skin with silk, using interrupted eversion mattress sutures.

FIG. 1:4 Insertion of ventricular guide tubes



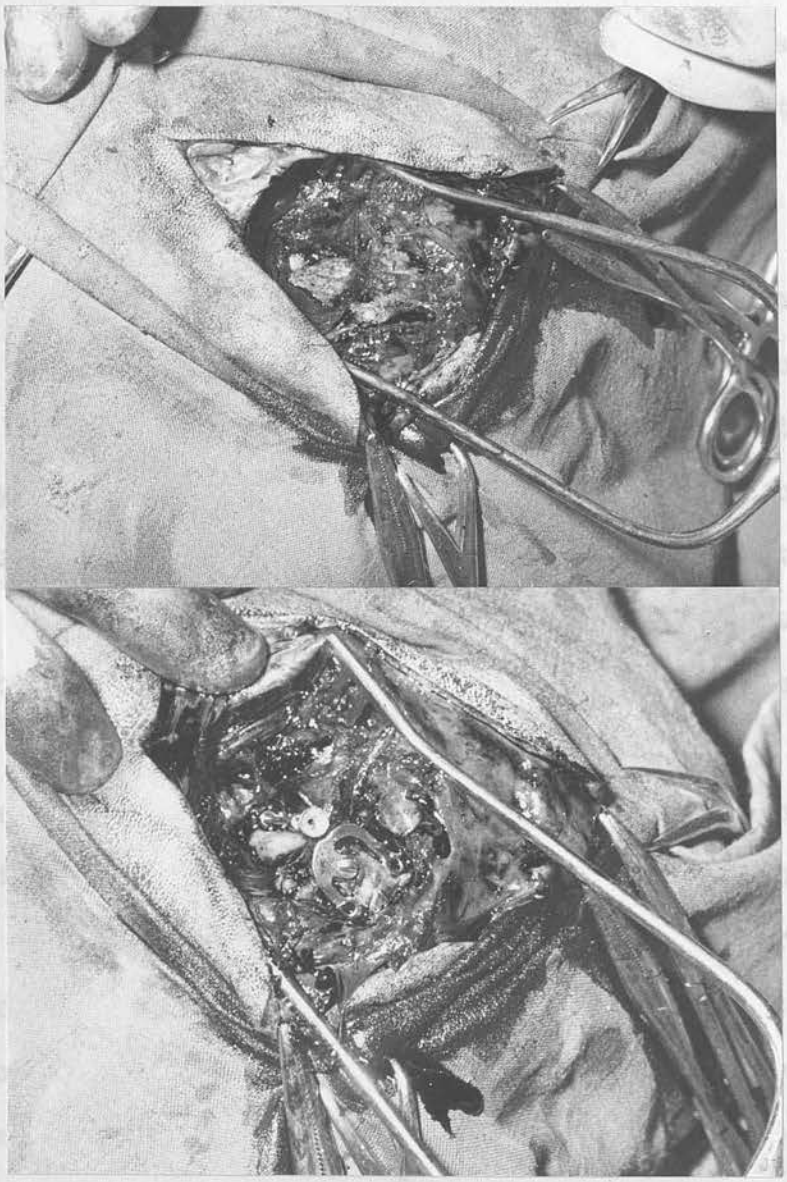
(Top) Operative exposure prior to insertion of the guide tube. Temporal muscles retracted to expose the temporal bone. The notch made in the sagittal crest marks the coronal plane used.

(Bottom) Guide tubes in place. The needle demonstrates that a free flow of c.s.f. can now be obtained from the lateral ventricle.

For insertion of the guide tubes to the cisterna magna (Fig. 1:3) the sterile towels were re-sited to expose the back of the skull and the neck, while the dog's nose was depressed by the assistant to bring the back of the head into the horizontal plane. An incision was then made in the skin starting 2 cm lateral to the midline at the level of the external occipital protruberance, and running caudally for 4 or 5 cm. This incision was retracted into the midline and the faint white nuchal ligament was located. This ligament and the underlying muscles were then incised longitudinally and retracted back from the midline by a self-retaining retractor. By careful blunt dissection the incision was deepened to expose the occipital bone from the external occipital protruberance right down to the atlanto-occipital membrane. This accomplished, the muscles and ligaments covering the external occipital protruberance were reflected with the periosteal elevator (Fig. 1:5).

A full length cisternal guide tube was then cut back until, with the horse shoe-shaped plate fitting snugly round the external occipital protruberance, the foot of the tube, with its collar, just rested on the atlanto-occipital membrane at the point where it emerged from under the occipital bone. In some of the dogs the occipital bone was found to form a small lip over the membrane and it was necessary to drill a small hole (1 mm diam.) through this to allow the guide tube a direct approach to the membrane. When this procedure was adopted the small collar which was usually

FIG. 1:5 Insertion of cisternal guide tube



(Top) Operative exposure prior to the insertion of the guide tube. The nuchal muscles have been split in the midline and retracted to expose the atlanto-occipital membrane and occipital bone. The occipital protuberance and adjoining region have been cleared of muscular attachment.

(Bottom) Guide tube in place. The tip of the tube rests in a hole drilled in the lip of the occipital bone. The top bracket is screwed firmly to the occipital protuberance by three screws.

placed at the end of the tube was raised 2 mm so that it rested not on the membrane, but on the bone.

After the length and positioning of the guide tube had been determined, it was held firmly in place by an assistant, while holes (1 mm diam.) were drilled through the plate into the bone of the external occipital protruberance. The holes were then tapped with an 8Ba tap and the plate firmly fixed to the skull with three stainless steel screws. Thus the tube was stabilised in position (Fig. 1:5) and since the correct siting could be verified by eye there was no need to carry out a test puncture.

Finally the muscles were brought together in a continuous blanket suture with 3.0 chromic gut and a similar suture was employed to close the subcutaneous fascia over the guide tube funnel. The skin incision was closed with silk thread using interrupted everting mattress sutures.

Sterile dressings were placed over the wounds and lightly bandaged into position, taking care not to obstruct the airway. The syringe containing the anaesthetic was removed from the vein and the endotracheal tube withdrawn. A special protective collar was placed over the dog's head to prevent its disturbing the bandages, and it was returned to a recovery kennel where the temperature was maintained at 24°C, until it had recovered from the anaesthetic. To minimise infection the dogs were given prophylactic antibiotics (1 ampoule crystamycin (Glaxo) - 1,000,000 units benzyl penicillin and 0.5 g streptomycin - intramuscularly once daily).

Perhaps the best explanation of the operation is provided by Figs. 1:6, 1:7 and 1:8 which show various views of a dog's skull with the guide tubes and cannulation needles in position.

Post Operative

Seven days after the operation the skin sutures were removed. If this proved in any way painful to the dog the procedure was carried out under light sodium thiopentone anaesthesia. The protective collar was left in place for a few days as a further precaution against the dog scratching the freshly healed wound.

Four weeks after the operation, when all the tissue reaction had subsided, tests were carried out to verify correct placement of the guide tubes, and determine the lengths of needles required for cannulation of the ventricular and cisternal spaces. The dog was anaesthetised with intravenous sodium thiopentone and a 21 gauge stainless steel needle was pushed through the skin of the dog's head and into one of the ventricular guide tubes. The needle was then slowly advanced down the guide tube and into the ventricle. Placement of the needle in the ventricle could be established by carefully withdrawing c.s.f. into a syringe under slight negative pressure. The length of needle required to penetrate to the ventricle was noted and the needle was then withdrawn. A similar procedure was carried out for the other two guide tubes and subsequently a set of three appropriately cut needles was prepared for the sampling or perfusion of that particular dog.

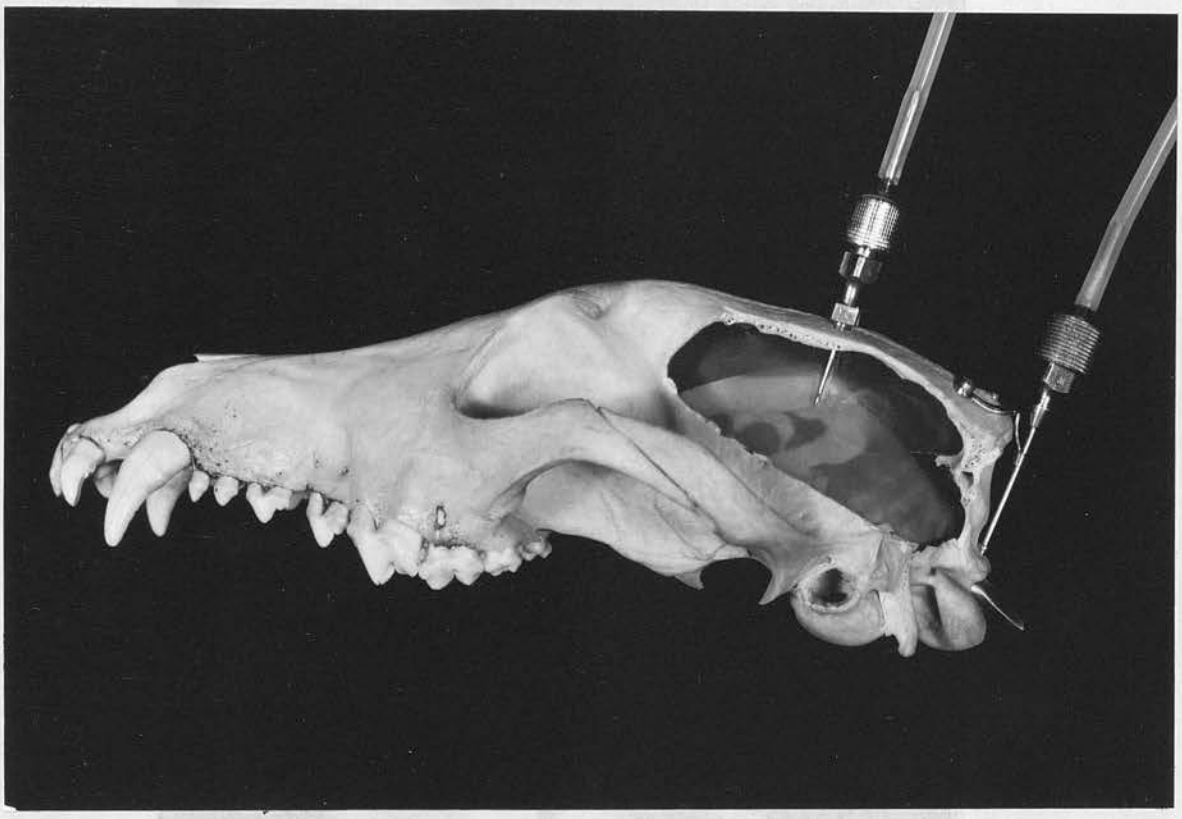


FIG. 1:6 Guide tubes inserted into a dog skull (lateral view)

An x-ray showing the ventricular system after an intraventricular injection of Myodil has been inserted into the appropriate position in the skull. The photograph demonstrates the position of the guide tubes and needles during perfusion.

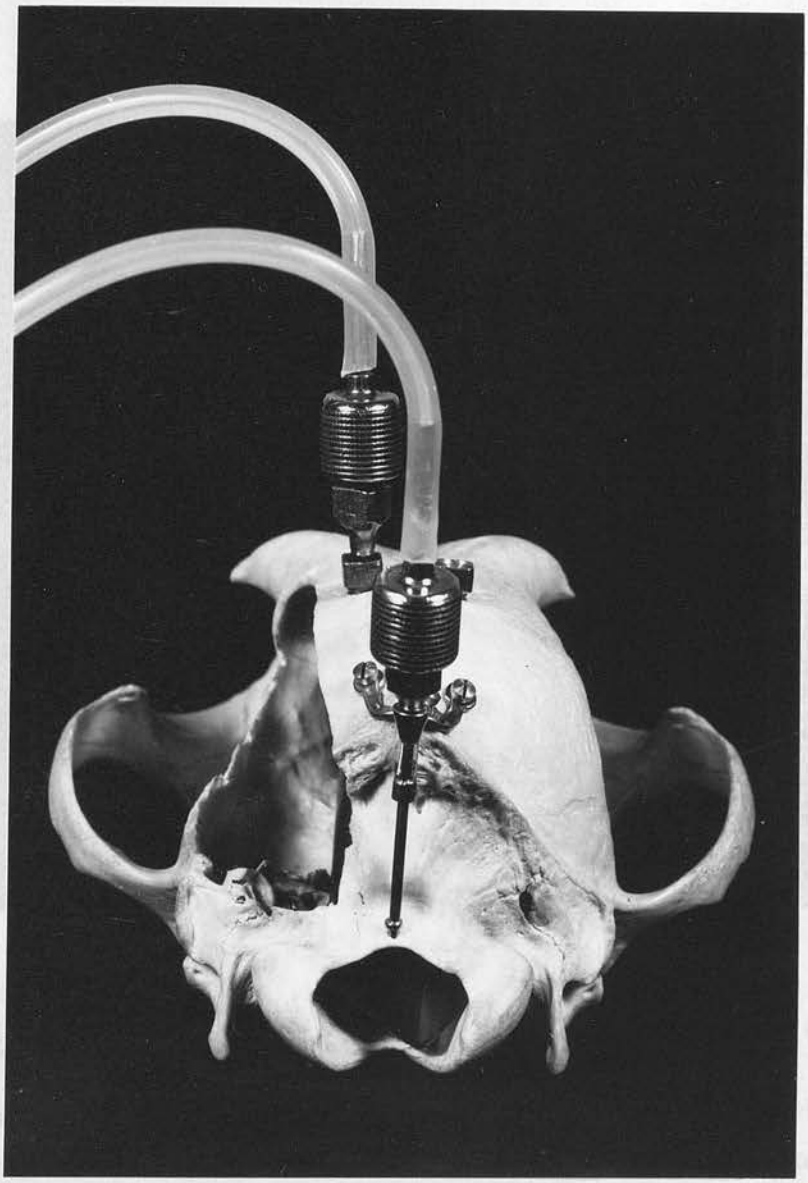


FIG. 1:7 Guide tubes inserted into a dog skull (posterior view)

The posterior view demonstrates how the cisternal needle is held in position. In this case it has been necessary to drill through a lip of the occipital bone to allow the needle direct access to the atlanto-occipital membrane.

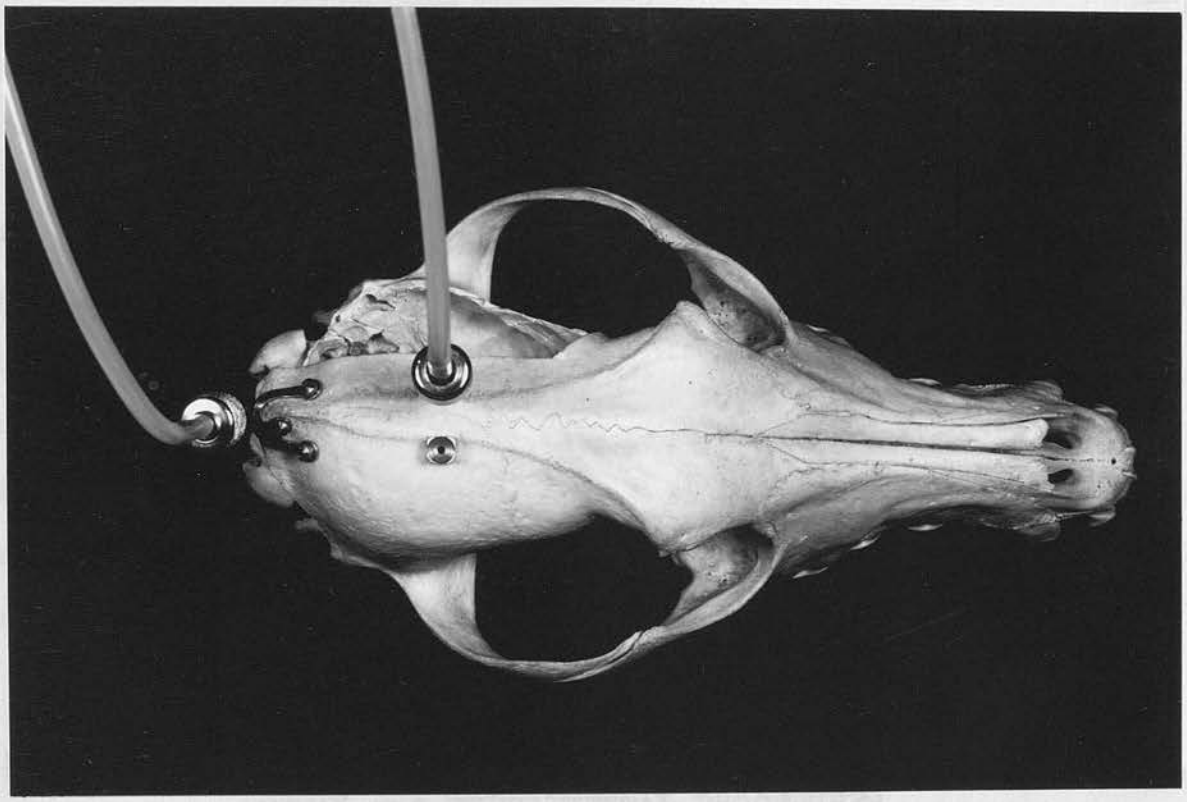


FIG. 1:8 Guide tubes inserted into a dog skull (Superior view)

The superior view demonstrates the positioning of the ventricular guide tubes and the method of fixation of the cisternal guide tube.

After a further two weeks the dog was considered to be ready for experimental use and it could be perfused up to once every two weeks or sampled two or three times a week.

Drugs and Chemical Reagents Used in Animal Experiments

Sodium thiopentone (Pentothal)	Abbot Laboratories
Heparin (1,000 i.u./ml)	Boots
Diphenylhydantoin (Epanutin)	Parke Davis
Carbamazepine (Tegretol)	Geigy
Sulthiame (Ospolot)	FBA Pharmaceuticals
Phenobarbitone	May and Baker
Ascorbic acid	B.D.H.

ANIMAL and EXPERIMENTAL PROCEDURES

4 adult male Beagle dogs with permanently implanted ventricular and cisternal guide tubes were used in these studies. Throughout the period of the experiment the dogs were kept on a standard batch prepared diet (Pedigree Chum, Petfoods Ltd.) and any sampling of body fluids was carried out between 09.30 and 12.00 hours.

Diphenylhydantoin (7.5 mg/kg/day), phenobarbitone (15 mg/kg/day), sulthiame (15 mg/kg/day) and carbamazepine (15 mg/kg/day) were administered orally in tablet or capsule form at 09.00 and 17.00 hours. The doses chosen were calculated to be equivalent

on a weight basis to the doses used to control epilepsy in humans.

After a period to establish control values for the parameters being measured (folate activity in c.s.f. and plasma; HVA and 5-HIAA concentrations in ventricular and cisternal c.s.f.), each of the dogs was put on one of the above drugs for a period of five weeks.

Standard sampling procedure was as follows. First the dog was anaesthetised with a minimal amount of intravenous sodium thiopentone and laid on its side. A 21 gauge needle was then inserted percutaneously into one of the ventricular guide tubes, advanced to the predetermined depth (see page 28) and c.s.f. was then slowly withdrawn, under slight negative pressure, into a disposable polyethylene syringe. The amount which could be withdrawn from one ventricle on any one occasion varied from dog to dog but since 0.5 ml was required for a folate assay, and at least 0.2 ml for estimation of the acid metabolites of the amines (HVA and 5-HIAA), it was normal practise to withdraw between 0.7 and 1.0 ml. If the volume from one ventricle was insufficient the other ventricle could also be sampled. After the required volume had been withdrawn the needle was removed from the syringe, and the c.s.f. gently expelled into a 15 ml glass centrifuge tube.

During sampling from the cisterna magna, light anaesthesia was maintained and the dog's head was flexed forward acutely.

A 21 gauge needle was then inserted percutaneously through the atlanto-occipital membrane (note - without using the guide tube to the cisterna magna) and 2.5 ml of cisternal c.s.f. was withdrawn slowly into a disposable polyethylene syringe. After the required volume had been removed the needle was gently withdrawn and the c.s.f. expelled into a 15 ml glass centrifuge tube. If the c.s.f. samples showed any trace of contamination by blood they were centrifuged at 1,300 g for five minutes at room temperature to remove the erythrocytes. This was necessary since haemolysis products interfere with HVA assays giving erroneously low values. Also, since erythrocytes contain large amounts of folate derivatives significant contamination of c.s.f. by blood can give erroneously high values for c.s.f. folate activity.

For a folate assay 0.5 ml of the c.s.f. sample was treated with 5 mg of ascorbic acid to give a 10 mg/ml solution with respect to ascorbate. This sample was then labelled and stored at -20°C until the assay could be performed.

For HVA and 5-HIAA assay an aliquot of between 0.2 and 0.5 ml for ventricular c.s.f., or 1.5 - 2.5 ml for cisteral c.s.f., was pipetted straight into a clearly marked 35 ml glass centrifuge tube. This tube was then stoppered and stored at -20°C until assay.

When a blood sample was required it was withdrawn from any suitable vein, other than that by which the anaesthetic was being administered. The vein was penetrated percutaneously with a

21 gauge needle and 5 ml of blood slowly taken up into a 10 ml disposable polyethylene syringe which contained 0.1 ml of heparin solution (1,000 I.U./ml). The needle was then withdrawn from the vein and the syringe inverted gently a few times to heparinise the blood thoroughly. The heparinised blood was then centrifuged at 1,300 g for 10 minutes. 2.5 ml of the plasma fraction were carefully removed and placed in a small glass vial. 25 mg Ascorbic acid was then added to the plasma and the sample was labelled and stored at -20°C until assay.

BIOCHEMICAL ANALYSES

Estimation of Homovanillic Acid (HVA) and 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) in Cerebrospinal Fluid.

The method used to measure the concentrations of homovanillic acid (HVA) and 5-hydroxyindol-3-ylacetic acid in the cerebrospinal fluid (c.s.f.) was essentially that of Ashcroft, Crawford, Dow and Guldberg (1968).

Materials

Authentic homovanillic acid and 5-Hydroxyindol-3-ylacetic acid were obtained from Koch-Light Co. Ltd.

All other reagents were obtained from British Drug Houses (B.D.H.) and were Analar grade, except for the ammonia solution and the hydrochloric acid which were both Aristar grade.

A fresh bottle of ethyl acetate (500 ml) was opened for each assay.

Distilled deionised water was used throughout, and all extractions and reactions were carried out in glass tubes fitted with glass stoppers.

Standard solutions and buffers were prepared as follows:-

Stock solutions of HVA and 5-HIAA

Solutions containing 1 mg/ml of HVA and 5-HIAA in water were stored at 4°C for periods of up to 3 weeks after which time they were renewed. During a three week period there was no detectable deterioration of the pure substances under these conditions.

Borate Buffer

0.1 M boric acid adjusted to pH 8.6 with 0.1 M sodium hydroxide solution.

Phosphate Buffer

0.1 M potassium dihydrogen orthophosphate adjusted to pH 7.4 with 0.1 M disodium hydrogen orthophosphate.

Spectrofluorimetry

The fluorescence measurements were made on an Aminco-Bowman Spectrophotofluorimeter with slit arrangement No. 5 (Aminco-Bowman Manual). Solutions whose fluorescence was to be measured were placed in 3 ml capacity Spectrosil cells (Scientific Supplies) with a 10 mm square internal cross section. For a true fluorescence reading the minimum volume of solution which could be used was 1.2 ml.

Extraction of Phenolic Acids

A measured volume of c.s.f. of between 0.2 ml and 1.0 ml for ventricular samples, and 1.5 and 2.0 ml for cisternal samples, was pipetted into a 35 ml glass centrifuge tube and the volume made up to 5.0 ml with water. The sample was then salt saturated with approximately 2 g of sodium chloride and taken to pH 1-2 with 25 µl of concentrated hydrochloric acid. 10 ml of ethyl acetate was added and the tube stoppered and shaken vigorously for 5 minutes. The mixture was then centrifuged at 1,900 g for 5 minutes to separate the two phases and 9.0 ml of the upper organic phase containing the extracted acids were withdrawn and pipetted into a fresh tube. Another 10 ml of ethyl acetate were added to the aqueous residue and the extraction repeated. This time however 10 ml of the organic phase were withdrawn and they were pooled with the first 9 ml giving 19 ml of ethyl acetate extract. The way in which this pooled extract was split for HVA and 5-HIAA assays depended on the estimated content of these acids in the original sample. In normal dog ventricular c.s.f. the concentration of HVA is much higher than that of 5-HIAA and a pooled ethyl acetate extract of such a sample was therefore divided to give 4 ml of extract for HVA assay and 15 ml for the 5-HIAA assay. The pooled ethyl acetate extract from cisternal samples was divided into two equal 9.5 ml fractions for the assay of HVA and 5-HIAA.

Estimation of 5-HIAA

The method used was a modification of that described by Ashcroft and Sharman (1962).

The ethyl acetate extract (15 ml for a ventricular sample and 9.5 ml for a cisternal sample) was shaken for 5 minutes with 1.4 ml 0.1 M phosphate buffer, pH 7.4, and the mixture centrifuged at 1,900 g. for 5 minutes. 1.0 ml of the lower aqueous phase was then carefully withdrawn and pipetted into a 15 ml glass test tube. This solution was then acidified with 0.4 ml concentrated HCl containing 50 mg% w/v ascorbic acid, and transferred to a cell for fluorimetry. The fluorescence of the hydroxyindol group was measured with an activation wavelength of 310 mμ and a fluorescence wavelength of 550 mμ. A yellow filter with a lower cut-off of 420 mμ was placed between the fluorescence monochromator and the photomultiplier in order to cut out a secondary scatter peak.

The fluorescence from the c.s.f. samples was compared with the fluorescence of standards of 5-HIAA, made up by diluting known aliquots of the stock solution in phosphate buffer, and acidifying with concentrated HCl containing ascorbate in exactly the same way as was done for the c.s.f. samples.

Estimation of HVA

The method used was essentially that of Andén, Roos and Werdinius (1963) as applied by Ashcroft, Crawford, Dow and Guldberg (1968) except that as in the 5-HIAA assay the tris

buffer was replaced by a buffer giving a lower blank. The phosphate buffer which was used in the 5-HIAA assay was not suitable since it caused quenching of the HVA fluorophor, and a borate buffer was found to be more satisfactory (Pullar, 1971).

The ethyl acetate extract (4 ml for a ventricular sample or 9.5 ml for a cisternal sample) was shaken for 5 minutes with 1.4 ml 0.1 M borate buffer pH 8.6 and the mixture centrifuged at 1,900 g. for 5 minutes. 1.0 ml of the lower aqueous phase was then carefully withdrawn and pipetted into a 15 ml glass test tube. To this were added 1.0 ml 5N ammonia solution and 0. 2 ml of freshly prepared potassium ferricyanide solution (10 mg% w/v). After exactly 4 minutes the reaction was terminated by the addition of 0.2 ml of freshly prepared L-cysteine solution (100 mg% w/v). The fluorophor derived from HVA was then measured at an activation wavelength of 325 mμ and a fluorescence wavelength of 430 mμ. These settings were found to give maximum readings for the fluorophors derived from standards of authentic HVA. As in the 5-HIAA assay the fluorescence of the c.s.f. samples was compared with the fluorescence of standard solutions of HVA in borate buffer after they had been treated in exactly the same way as the borate buffer extracts of the c.s.f. samples.

Recoveries and blanks

In order to determine the efficiency of the extraction of the acids, known amounts of the pure substances were made up in 5 ml of water in 30 ml glass centrifuge tubes and then taken

through the whole procedure exactly as if they were c.s.f. samples. By comparing the final fluorescence with standards of HVA and 5-HIAA it was possible to calculate a recovery figure which could then be applied to the c.s.f. samples to calculate the absolute concentrations of these acids in the c.s.f.

"Extraction Blanks" were obtained by taking 5 ml of water through the whole procedure and treating the appropriate buffer extracts with the fluorophor-producing reagents.

Normal assay procedure

The most economical way of assaying a large number of c.s.f. samples was to run them through the whole procedure in batches, along with tubes for blanks and recoveries. The assay plan is shown in Table 1:2.

At the stage of splitting the ethyl acetate extract (see page 37) tubes 1-7 were treated as if they contained ventricular c.s.f. samples, while tubes 8-14 were split as if they contained cisternal c.s.f. samples. The mean recovery figures for tubes 2 and 7 were then directly applicable to the ventricular c.s.f. samples in tubes 3-6 while the mean recoveries for tubes 9 and 14 were directly applicable to the cisternal c.s.f. samples in tubes 10-13.

TABLE 1:2 : Protocol for assay of HVA and 5HIAA in c.s.f. samples

1. Blank
2. Recovery : 2 µg HVA and 100 ng 5-HIAA
3. Ventricular c.s.f. sample
4. "
5. "
6. "
7. Recovery : 1 µg HVA and 200 ng 5-HIAA
8. Blank
9. Recovery : 2 µg HVA and 100 ng 5-HIAA
10. Cisternal c.s.f. sample
11. "
12. "
13. "
14. Recovery : 1 µg HVA and 200 ng 5-HIAA.

ESTIMATION OF FOLATE IN PLASMA AND CEREBROSPINAL FLUID

Introduction

Although folic acid and its derivatives can be estimated colourimetrically after a diazotisation procedure (Hutchings, Stokstad, Boothe, Mowat, Waller, Angier, Semb and Subbarow, 1960), these methods are not sensitive enough to assay the levels

found in plasma and c.s.f. For this reason we used a micro-biological assay technique based on the principle that the amount of folate in a sample can be determined from the growth response of a folate-dependent micro-organism when an aliquot of the sample is added to a folate free, but otherwise nutritionally adequate, culture of the micro-organism.

The method used was essentially that of Waters and Mollin (1961) and makes use of a folate-dependent strain of Lactobacillus casei. This is the least discriminating of the normal test organisms in that it can utilise all the biological forms of folate except those which are conjugated with three or more glutamic acid residues.

Materials

The test micro-organism, Lactobacillus casei, strain NCIB 8010, was obtained from a stock held in the Department of Therapeutics, Royal Infirmary of Edinburgh.

Folic acid and L-tryptophan were obtained from Koch-Light Co., Ltd. and the basic folic acid-L. casei culture medium (Baker, Herbert, Frank, Pasher, Hutner, Wasserman and Sobotka, 1959) was obtained from Difco Laboratories.

All other reagents were Analar grade (B.D.H.) or equivalent quality and distilled water was used throughout.

Stock solutions and buffers were prepared as follows:-

Folic acid standard solution

This was prepared freshly for each assay by two

consecutive 1:200 dilutions of a 40 µg/ml stock solution to give a solution of 1 ng/ml folic acid. The stock solution was kept in a dark bottle at 4°C.

L. casei medium

This was prepared by adding l-tryptophan to the basic Difco folic acid-L. casei medium (10 mg tryptophan/100 ml basic medium) (Ball and Giles, 1964).

Phosphate buffer

0.1 M sodium dihydrogen orthophosphate adjusted to pH 6.1 with 0.1 M disodium hydrogen orthophosphate. Note that before use as a serum diluent this buffer was supplemented with ascorbic acid, 200 mg/100 ml.

PLASMA ASSAY

The sample of ascorbate treated plasma (see p. 35) was thawed out and 0.7 ml pipetted into a glass universal tube and diluted with 6.3 ml 0.1 M phosphate buffer containing ascorbic acid (200 mg/100 ml). The sample was then autoclaved at 15 lbs./sq.in. for 2.5 minutes to precipitate protein. After cooling, the tube was centrifuged at 1,000 g. for 5 minutes and the supernatant filtered through Whatmans No. 40 paper. 2.5 ml of the filtrate were then made up to 10 ml with water and 2 ml aliquots of this diluted plasma extract were added to 4 test-tubes, each containing 2 ml of L. casei medium.

Standards to cover the range of 0-1.4 ng folate per tube were prepared in duplicate by adding appropriate volumes of the I ng/ml folic acid standard solution to tubes containing 2 ml of medium and then making the volume up to 4 ml with water. The normal practise was to prepare duplicate sets of standard tubes containing respectively 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 ng folic acid. In addition an extra "blank" tube containing 2 ml of medium and 2 ml of water was prepared. At a later stage in the assay when the standard and plasma tubes were inoculated with the *L. casei* micro-organism this tube remained uninoculated and it was subsequently used as the "blank" in the turbidity measurements.

Standard and plasma tubes along with the "blank" were autoclaved at 10-12 p.s.i. for 10 minutes. After this sterilisation procedure the tubes were allowed to cool down; then all, except the blank, were inoculated with the folate-dependent strain of *L. casei*. The tubes were then incubated at 37°C for 18-30 hours.

The end point of the assay was set at the time when the standard tubes containing 0.6 ng of folic acid developed an optical density of 0.156. The optical density measurements were carried out on a Spekker absorptiometer by reading the samples against the uninoculated "blank" at filter setting No. 3 (460 - 530 mu). Under these conditions the optical densities of the solutions were directly related to their turbidities. When the end point of the assay was reached all the samples were

removed from the incubator and their optical densities were measured. A standard curve of optical density against nanograms of folate per tube was then drawn up (Fig. 1:9), and this was used in the calculation of the folate concentration in each plasma tube. This value was then multiplied by the dilution factor of 80 to give the plasma folate concentration in nanograms of folate per ml of plasma.

In this assay each sample of plasma gave rise to 4 different estimates of the plasma folate concentration. Unless there was an obvious error in any of the estimations the mean of the 4 values was accepted as the best estimate.

C.S.F. ASSAY

The c.s.f. assay was basically similar to the plasma assay except that there was no necessity for an initial protein precipitation step. In order to bring the folate activities of the c.s.f. samples within the sensitive range of the standard curve it was necessary to assay c.s.f. at higher dilutions than those which were employed in the assay of plasma samples.

The ascorbate treated c.s.f. sample (see page 34) was thawed out, and an 0.2 ml aliquot diluted to 10 ml with 1% (w/v) ascorbic acid solution. A further 0.2 aliquot of c.s.f. was then diluted with 19.8 ml 1% (w/v) ascorbic acid solution and 10 ml of the resultant solution removed to another tube and there diluted with 5 ml 1% (w/v) ascorbate solution.

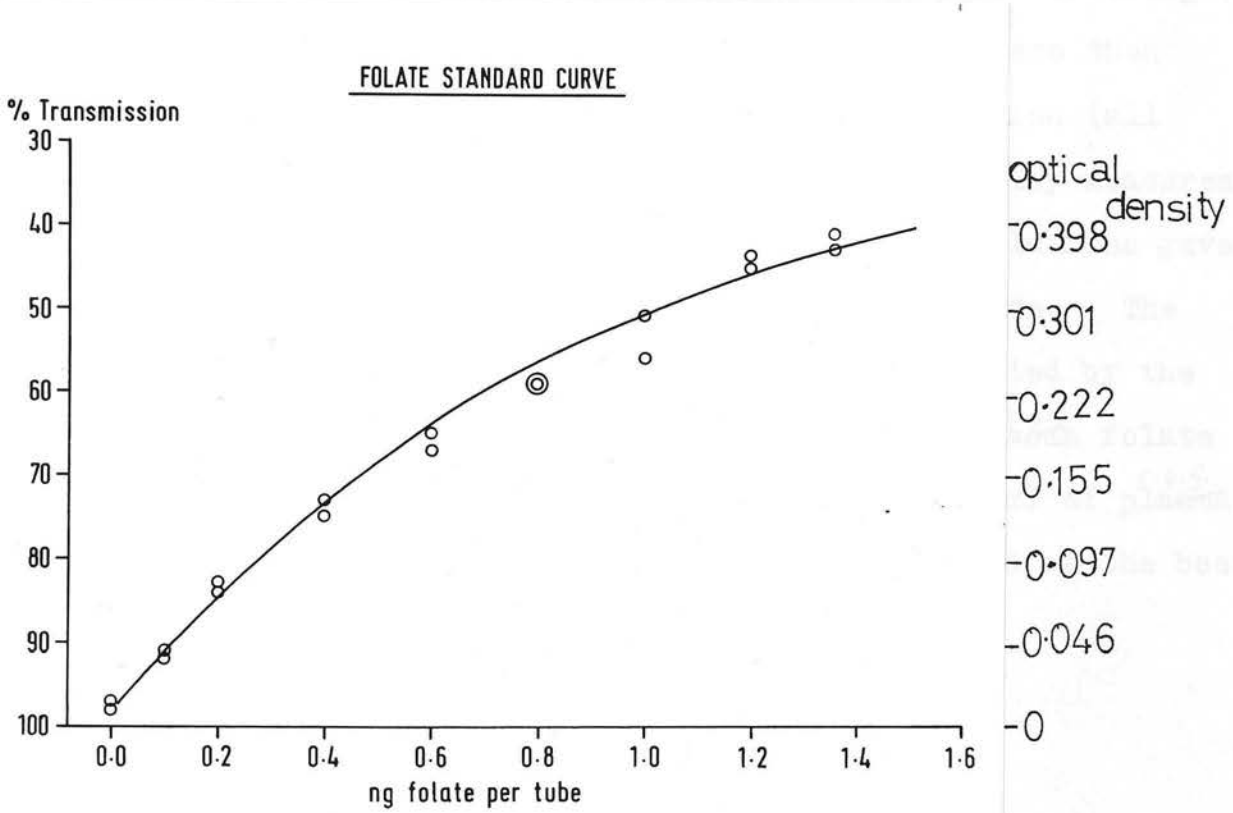


FIG. 1:9 Standard growth curve for folate-dependent l. casei

The optical density (460-530 mμ) of a solution after incubation (see text pp 43-45) is a measure of the rate of growth of l. casei in that solution.

Four 2 ml aliquots of each of these three c.s.f. dilutions (1:50, 1:100, 1:150) were then added to tubes containing 2 ml of medium, giving in all, 12 tubes per c.s.f. sample. These tubes, along with a "blank" and duplicate standards covering the range 0-1.4 ng/tube, as in the plasma assay, were then taken through the steps of sterilisation, inoculation (all except the "blank"), incubation and finally turbidity measurement.

As a rule only one, or perhaps two, of the dilutions gave readings on the sensitive part of the standard curve. The estimates from satisfactory dilutions were multiplied by the appropriate dilution factors to give values for ~~plasma~~ c.s.f. folate concentration in terms of nanograms of folate per ml of ~~plasma~~ c.s.f.. The mean of all satisfactory estimates was accepted as the best result.

RESULTS

Pre-Treatment Control Studies

Assays on samples taken from the dogs before anticonvulsant treatment was started revealed that the folate activities of c.s.f. and plasma in the dog were similar to those reported for man (Table 1:3). The mean c.s.f. folate concentration found in this study on the dog was rather higher than the values previously reported for human lumbar c.s.f. (Table 1:3; Wells and Casey, 1967; Jensen and Olesen, 1971). However it is difficult to say whether this is a true difference since in fact controls from a later study with the same dogs (Section 1 B; see

TABLE 1:3 : Folate Activity in Body Fluids of Dog and Man

	Plasma or Serum	c.s.f.
DOG	12.2 (6.2 - 25.0)[0]	41.5 (10 - 70)[1]
MAN	9.9 (5.9 - 20)[2]	20.9 (9 - 38)[3]
	C.S.F./Plasma Folate Ratio	
DOG	1.9 - 7.1	
MAN	3.0[4]	

[0] - mean (range) (ng/ml)

[1] - values for combined ventricular and cisternal c.s.f. estimations.

[2] - serum Waters and Mollin (1961)

[3] - lumbar c.s.f. Reynolds, Preece and Chanarin (1969)

[4] - mean reported by Herbert and Zalusky (1961).

page 73) displayed much lower c.s.f. folate activities. In the dog, the ratio of folate activity between c.s.f. and plasma ranged from 1.9 - 7.1 as compared with a mean ratio of 3.0 which Herbert and Zalusky (1961) reported for man.

Because of the similarities between plasma and c.s.f. folate activities in the dog and in man, both in relative and absolute terms, it seems reasonable to assume that the dog is a good test animal for studies of folate distribution which may be capable of extrapolation to man.

Dogs A1 (Fig. 1:10), A2 (Fig. 1:11) and A3 (Fig. 1:12) all displayed a positive correlation between the folate activity of plasma samples and the corresponding samples of c.s.f. obtained at the same time ($p < 0.05$). A similar result has been reported for studies in man by Wells and Casey, 1967, Reynolds, Preece and Chanarin, 1969 and Jensen and Oleson, 1971). The ventricular and cisternal values have been plotted separately since a very slight but significant difference was found (ventricular cisternal : mean difference 5.2 ng/ml : $p < 0.001$) when carrying out a paired t-test on samples which had been taken at the same time. Plotting the values separately in this fashion demonstrated an interesting trend in that the three dogs which displayed a positive correlation between c.s.f. and plasma folate all showed a greater regression coefficient on ventricular c.s.f. samples.

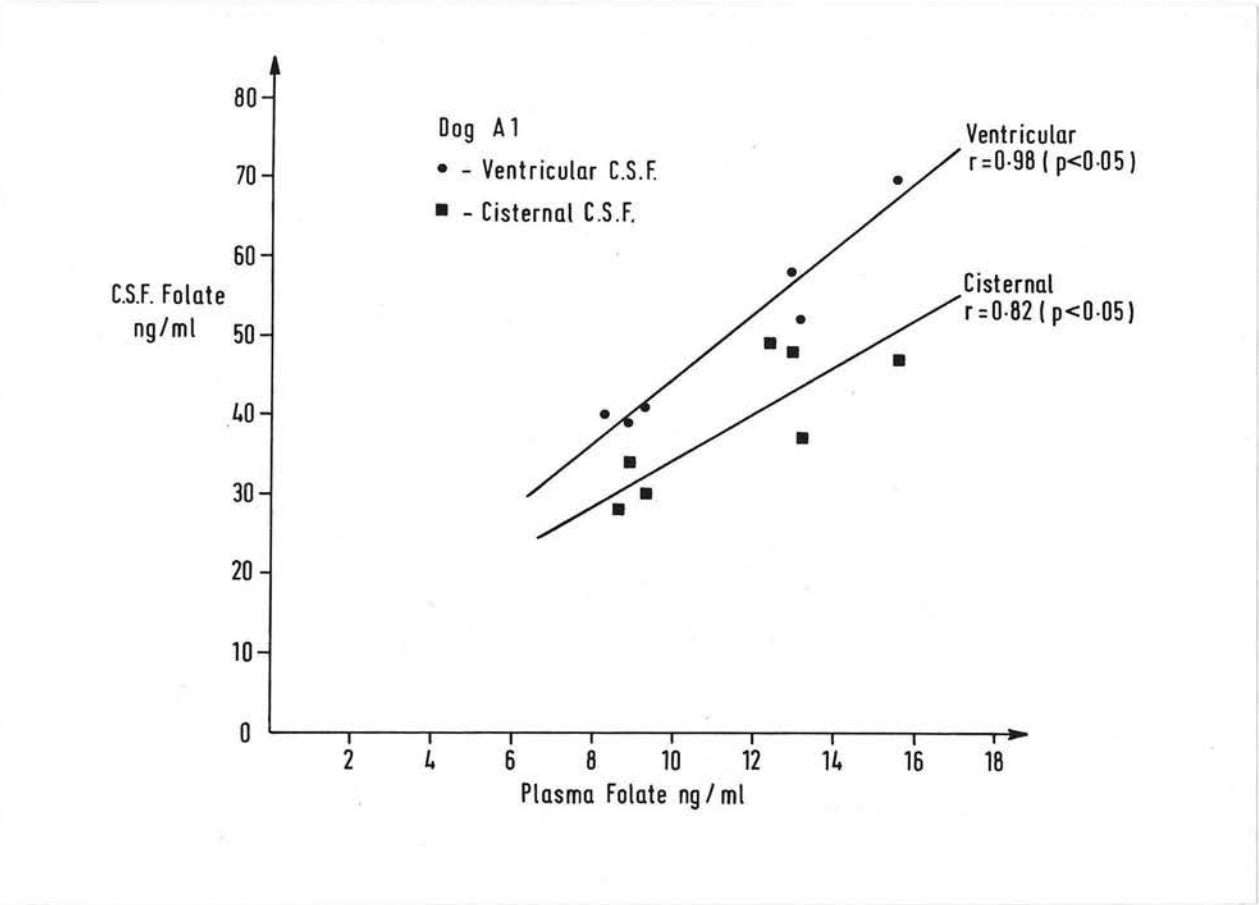


FIG. 1:10 Correlation between c.s.f. and plasma folate activity in dog A1. The points are derived from paired samples of c.s.f. and blood collected during the control period.

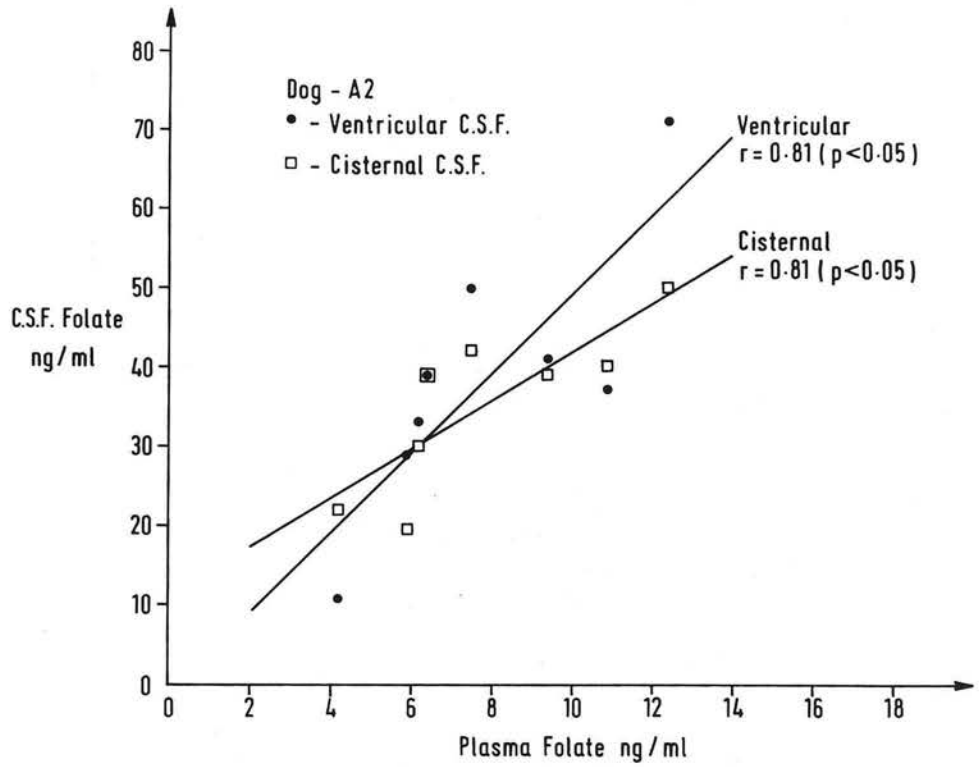


FIG. 1:11 Correlation between c.s.f. and plasma folate activity in dog A2. The points are derived from paired samples of c.s.f. and blood collected during the control period.

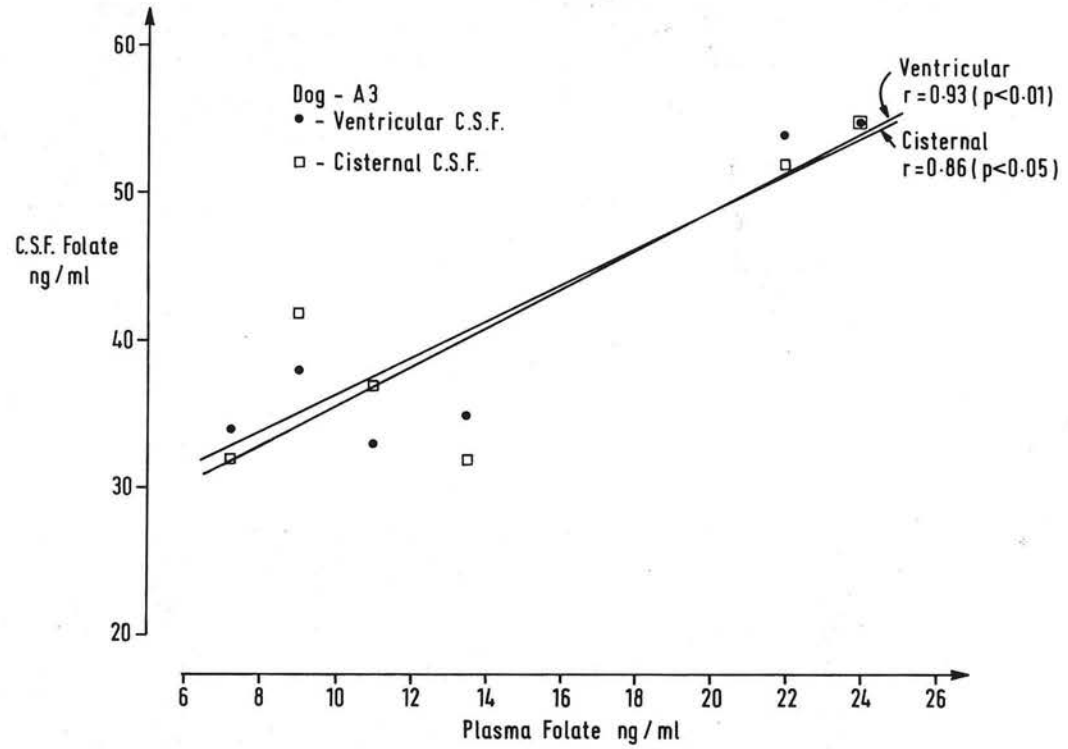


FIG. 1:12 Correlation between c.s.f. and plasma folate activity in dog A3. The points are derived from paired samples of c.s.f. and blood collected during the control period.

Anticonvulsant Treatment

As can be readily seen from Tables 1:4-7 none of the anticonvulsants carbamazepine (15 mg/kg/d), diphenylhydantoin (7.5 mg/kg/d), phenobarbitone (15 mg/kg/d) or sulthiame (15 mg/kg/d) in the time for which the drugs were administered (36 days), had any detectable effect on the folate activities of either c.s.f. or plasma. This is perhaps not unexpected with sulthiame and carbamazepine, since neither of these drugs have been reported to reduce folate concentrations in man. The lack of effect of diphenylhydantoin and phenobarbitone suggests either that their folate depleting actions may only be obvious after a long period (years) of treatment, or that there is a species difference between man and dog with respect to anticonvulsants and folate metabolism.

HVA and 5-HIAA studies

Pre-treatment control studies confirmed the presence of a sharp gradient in the concentrations of both these acids between the ventricle and the cistern (Guldberg, Ashcroft and Crawford, 1966), which is due to a localised transport system for the removal of these metabolites in the region of the fourth ventricle (Ashcroft, Dow and Moir, 1968). Treatment with the anticonvulsants had no detectable effect on the HVA and 5-HIAA concentrations in the lateral ventricles or the cisterna magna (Tables 1:8-11). This was not unexpected in view of the lack of change in folate concentrations in the c.s.f. (Tables 1: 4 - 7).

TABLE 1:4 : Dog A1 - TREATMENT - CARBAMAZEPINE

	Day	Folate Concentration (ng/ml) C.S.F. Vent.	C.S.F. Cist.	Plasma
CONTROL	4	-	30	-
	8	58	48	13.0
	14	68	32	-
	16	-	49	-
	20	70	47	15.6
	22	-	49	12.4
	27	40	28	8.3
	29	41	30	9.3
	34	-	-	19.5
	36	54	37	13.2
	42	44	38	-
Mean ± S.D.		53.6 ± 12.5	38.8 ± 8.7	13.0 ± 3.8
TREATMENT	44	51	39	-
	50	-	-	8.2
	56	43	45	16.9
	58	51	40	7.6
	63	33	36	8.7
	79	59	40	10.3
Mean ± S.D.		47.4 ± 9.8	40.0 ± 3.2	10.3 ± 3.8
CONTROL	92	39	34	8.9

TABLE 1:5 : Dog A2 - TREATMENT - DIPHENYLHYDANTOIN

	Day	Folate Concentration (ng/ml) C.S.F. Vent.	C.S.F. Cist.	Plasma
CONTROL	1	10.8	22	4.2
	8	37	40	10.9
	14	-	47	-
	16	43	35	-
	20	71	50	12.4
	22	50	44	7.5
	27	29	19.6	5.9
	29	33	30	6.2
	34	-	-	10.4
	36	41	39	9.4
	42	36	22	-
Mean ± S.D.		39.0±16.2(9)	36.0±11.1(11)	8.4±2.8(8)
TREATMENT	44	34	32	6.1
	50	30	28	-
	56	35	38	6.1
	58	42	34	4.9
	63	43	28	7.5
	79	39	37	4.7
Mean ± S.D.		37.2±5.0(6)	32.8±4.3(6)	5.9±1.1(5)
CONTROL	92	38	38	6.4

TABLE 1:6 : Dog A3 - TREATMENT - PHENOBARBITONE

	Day	Folate Concentration (ng/ml) C.S.F. Vent.	C.S.F. Cist.	Plasma
CONTROL	20	55	55	24
	22	54	52	22
	27	31	32	-
	29	34	32	6.6
	34	35	32	13.5
	36	33	37	11.0
	42	35	30	-
Mean ± S.D.		39.6±10.3(7)	38.6±10.5(7)	15.4±7.4(5)
TREATMENT	44	45	42	10.3
	50	32	28	7.0
	56	39	38	9.4
	58	46	45	9.5
	63	27	38	15.4
	79	-	37	5.2
Mean ± S.D.		37.8±8.2(5)	38.0±5.8(6)	9.5±3.5(6)
CONTROL	92	38	42	9.0

TABLE 1:7 : Dog A4 - TREATMENT - SULTHIAME

	Day	Folate Concentration (ng/ml) C.S.F. Vent.	C.S.F. Cist.	Plasma
CONTROL	4	40	34	-
	8	48	47	25
	14	55	-	-
	16	48	45	-
	20	51	62	19.4
	22	57	52	11.6
	27	43	36	-
	29	39	36	12.4
	34	46	40	14.5
	36	42	44	15.0
	42	43	44	-
Mean ± S.D.		46.5±5.9(11)	44.0±8.4(10)	16.3±5.1(6)
TREATMENT	44	50	-	11.6
	50	31	-	11.8
	56	40	-	12.5
	58	56	56	11.2
	63	44	-	8.5
	79	56	-	-
Mean ± S.D.		46.2±9.8(6)		11.2±1.6
CONTROL	92	47	38	7.4

TABLE 1:8 : Dog A1 - TREATMENT - CARBAMAZEPINE

	Day	HVA Conc. in C.S.F. Vent.	HVA Conc. in C.S.F. Cist.	5-HIAA Conc. in C.S.F. Vent.	5-HIAA Conc. in C.S.F. Cist.
CONTROL	1	740	104	290	37
	15	790	103	250	56
	21	800	112	230	23
TREATMENT	28	1330	84	280	29
	31	920	158	200	27
Mean ± S.D.		1017±278(3)	118±38(3)	237±40(3)	26±3(3)

TABLE 1:9 : Dog A2 - TREATMENT - DIPHENYLHYDANTOIN

	Day	HVA Conc. in C.S.F. Vent.	HVA Conc. in C.S.F. Cist.	5-HIAA Conc. in C.S.F. Vent.	5-HIAA Conc. in C.S.F. Cist.
CONTROL	1	2280	222	278	43
	15	2060	121	300	40
	21	2100	133	308	29
TREATMENT	28	2680	108	318	37
	31	2320	107	199	16
Mean ± S.D.		2366±293(3)	116±15(3)	275±66(3)	27.3±11(3)

TABLE 1:10 : Dog A3 - TREATMENT - PHENOBARBITONE

	Day	HVA Conc. in C.S.F. Vent.	HVA Conc. in C.S.F. Cist.	5-HIAA Conc. in C.S.F. Vent.	5-HIAA Conc. in C.S.F. Cist.
CONTROL	1	2400	121	276	56
	15	2140	74	343	56
	21	2140	103	333	28
TREATMENT	28	2370	131	343	26
	31	2210	139	241	24
Mean ± S.D.		2240±118(3)	124.3±19(3)	306±56(3)	26±2(3)

TABLE 1:11 : Dog A4 - TREATMENT - SULTHIAME

	Day	HVA Conc. in C.S.F. Vent.	HVA Conc. in C.S.F. Cist.	5-HIAA Conc. in C.S.F. Vent.	5-HIAA Conc. in C.S.F. Cist.
CONTROL	1	2250	167	284	41
	15	2560	135	300	21
	21	2490	128	283	25
TREATMENT	28	2780	-	335	-
	31	2660	175	278	23
Mean ± S.D.		2643±333(3)	152±33(2)	299±32(3)	24±1.4(2)

Discussion

Many different theories have been proposed to account for the disturbances in folate metabolism brought about by chronic treatment with the anticonvulsants diphenylhydantoin phenobarbitone, and primidone. Before it was known that there was an actual decrease in folate concentration in plasma and c.s.f. Girdwood and Lenman (1956) suggested that the anticonvulsants, by virtue of their structural similarities to folates, might be inhibiting the activities of folate-dependent enzymes. Alternatively they might be blocking the interconversion of the various folate derivatives. However Klipstein (1964) showed that the anticonvulsants do not interfere with l.caseii assays and Hamfelt and Wilmans (1965) could not demonstrate any inhibitory effect on dihydrofolate reductase, methylene tetrahydrofolate dehydrogenase or formyl tetrahydrofolate synthetase.

When it became known that serum folates were reduced in anticonvulsant-induced anaemias Klipstein (1964) suggested that the anticonvulsants might be displacing folate from plasma proteins and thereby lowering total plasma concentrations. There has been no experimental proof to support this however, and no such effect was seen in the present study.

There is evidence that anticonvulsants interfere with the absorption of the folates derived from food. Hoffbrand and Necheles (1968) and Rosenberg, Godwin, Streiff and Castle (1968) both reported that anticonvulsants inhibited the intestinal

'conjugase' enzymes. As most of the folates present in food are in the polyglutamate form (Butterworth, Santini and Frommeyer, 1963) such an action could result in poorer utilisation of dietary folate since only non-conjugated derivatives appear to be actually absorbed (e.g. Butterworth, Baugh and Krumdieck, 1969). Both Hoffbrand and Nechelles (1968) and Rosenberg et al. (1968) reported that the absorption of folic acid itself was unaffected by anticonvulsants. However Meynell had reported in 1966 that the absorption of pure folic acid was reduced by an acute dose of diphenylhydantoin. Several subsequent studies have not only confirmed this latter finding but have been unable to find any effect of anticonvulsants on preparations of intestinal 'conjugase' enzymes (Baugh and Krumdieck, 1969; Bernstein, Gutstein, Weiner and Efron, 1970; Gerson, Hepner, Brown, Cohen, Herbert and Janowitz, 1970). These findings are exactly the reverse of those reported by Hoffbrand and Necheles, and Rosenberg et al.. However, whichever mechanism of action proves to be right, or indeed if both do, one might not expect anticonvulsant therapy to affect body stores of folate unless the dietary intake of folate was barely sufficient and anticonvulsants were administered in high dosage over a long time. If this were the only way in which anticonvulsants interfere with folate transport and metabolism there might be no measurable change in the folate activity of plasma and c.s.f. until the large tissue stores of folates

were considerably depleted (Herbert, 1962). This may be one reason why we saw no effect in this study after only 4 weeks of anticonvulsant treatment. Another possibility is that because of species differences in metabolism the dosage of anticonvulsants administered (equivalent to therapeutic doses in man) were not sufficiently high.

The effect of diphenylhydantoin on the transport of folates between the plasma and the c.s.f. is also the subject of some debate. Levitt, Pincus, Nixon and Bertino (1969) reported that methyl tetrahydrofolate is actively taken up into the c.s.f., and that diphenylhydantoin had no effect on this transport. However preliminary findings from Woodbury and Kemp (1971) indicate that the uptake of folic acid into all tissues, including the c.s.f., is inhibited by diphenylhydantoin. The contrasting conclusions of these two studies may be explained by the fact that the transport of different derivatives was being studied, but until more is known of the exact experimental procedures this can only be speculation.

When Reynolds (1967) observed an increase in frequency and severity of fits in patients on anticonvulsant therapy who were given folic acid this was taken as support for the hypothesis that the anti-folate effects of anticonvulsants were related to their therapeutic effects. However, there is evidence that the restoration of tissue folates results in more rapid metabolism of the anticonvulsant drug, to the extent of producing sub-

therapeutic plasma concentrations of the anticonvulsant (Baylis, Crowley and Preece, 1971; Oleson and Jensen, 1970). These findings have not been supported by Andreasen, Hansen, Skovsted and Nielsen (1971) who were unable to demonstrate any effect of folic acid treatment on the metabolic half life of diphenylhydantoin in man.

In conclusion therefore it can be said that there is very little evidence to support a causal relationship between the anti-folate effects of anticonvulsant drugs and their therapeutic actions, though there are certainly some interactions in man between prolonged anticonvulsant therapy and folate metabolism.

In view of the fact that the folate activities of c.s.f. and plasma were unaltered in this study, the absence of any change in the c.s.f. concentrations of the amine metabolites, HVA and 5-HIAA, was not unexpected. Even if the anticonvulsants had reduced the c.s.f. and plasma folate activities there is some evidence to suggest that the availability of co-factor for aromatic amino acid hydroxylation in the brain might not have been significantly affected.

Allen and Klipstein (1970) have reported that the brain's stores of folate are very much more resistant to depletion during the development of a folate deficiency state, than are those of the liver. More significantly Goodfriend and Kaufman (1961) found that there was very little reduction in the availability

of pteridine co-factor in the liver in even a severe folate deficiency state. These two findings suggest that the amine synthesising systems in the brain may be relatively "protected" during the development of a folate deficiency state. In this context it may be significant that in contrast to peripheral hydroxylation systems the enzyme and co-factor for brain tryptophan hydroxylation are very firmly bound to particulate matter (Grahame-Smith, 1967).

SECTION 1B: The Effect of Folic Acid Administration on (a) the Folate Activity of c.s.f., Plasma and Plasma Ultrafiltrate and (b) the Concentrations of HVA and 5HIAA in Ventricular and Cisternal c.s.f.

Introduction

While carrying out investigations on the inter-relations between folic acid and vitamin B12 metabolism Hunter, Barnes, Oakeley and Matthews (1970) administered large doses of folic acid (15 mg daily) to a group of healthy volunteers. As they report however, the trial "was abandoned after one month of a projected three month project because of the unexpected development of increasingly disturbing toxic effects in the majority. Most of the subjects experienced mental changes, sleep disturbances and gastrointestinal symptoms." The

mental changes which included altered sleep patterns, irritability, overactivity and excitement, suggested a rather non-specific heightening of mood and an increase in the general level of arousal. Since there was no concomitant change in vitamin B12 levels the authors deduced that the effects described were due specifically to the folic acid treatment. It was these findings of Hunter et al. which led to the work reported in this section of the thesis.

Since our own studies were started, Hunter et al.'s work has been much criticised for its uncontrolled nature (Snaith, 1970; Ralston, 1970; Standage, 1970; Davis and Woodliff, 1971; Gibberd, Nicholls, Dunne and Chaput de Saintonge, 1971), but both before and after this report there have been other independent studies which have implied a link between folic acid and mental function. Reynolds, Milner, Matthews and Chanarin in their 1966 paper put forward the hypothesis that drug-induced disturbances in folate and B12 metabolism may be concerned in the production of mental symptoms in epileptic patients. Reynolds et al. (1967) reported a marked improvement in drive in 22 out of 26 patients who were given folic acid to correct an anaemia induced by anticonvulsant therapy. Neubauer (1970) was of the opinion that folic acid was helpful in alleviating the mental retardation which can develop in drug-treated epileptics. On a broader theme Strachan and Henderson (1967) have described two patients with dementia who

improved when their low serum folates were restored to normal with folic acid treatment. Low serum folates have been reported in up to a quarter of psychiatric in-patients (Carney, 1967; Hunter, Jones, Jones and Matthews, 1967; Kallstrom and Nylöf, 1969; Reynolds, Preece, Bailey and Coppen, 1970) though, as Carney and Sheffield (1970) pointed out, a combination of many different factors may contribute to this finding. Mental retardation has been a feature of certain congenital metabolic disorders involving folate absorption (Lanzkowsky, 1970) and folate metabolism (Arakawa, 1970). Thus, despite the fact that several trials (Snaith, 1970; Ralston, Snaith and Hinley, 1970; Jensen and Oleson, 1970; Gibberd et al., 1971; Hellström, 1971) have not confirmed the effects of folic acid reported by Hunter et al. (1970) there is other evidence linking folic acid with mental function.

Hunter et al. (1970) hypothesised that the mental symptoms of "folic acid intoxication" observed by themselves, and the "alterting" effect of folic acid observed by Reynolds (1968), might be due to changes in brain amine synthesis. As indicated earlier (Section 1A, page 19) the rate limiting steps on the pathways leading to the formation of dopamine and 5-hydroxytryptamine, namely the hydroxylation of tyrosine and tryptophan respectively, are dependent on pteridine co-factors, (Nagatsu et al. 1964; Gal et al. 1968). Folates themselves form a class of substituted pteridines and it has been demonstrated

that certain folate derivatives can replace the natural pteridine co-factor in the aromatic amino acid hydroxylation systems of the liver (Kaufman, 1963). On the basis of the normal finding of very much higher folate activity in c.s.f. than in plasma, Hunter et al. had inferred that their volunteers on folic acid would have very high concentrations of folate in their c.s.f. If this were the case it might also mean that there was an increase in the availability of co-factor for the rate-limiting steps in brain amine synthesis. This seemed a reasonable hypothesis since the c.s.f. is known to be very similar in composition to brain extracellular fluid (see review - Davson, 1968). Such a hypothesis could also provide an explanation for the mental changes observed after folic acid treatment since aminergic systems in the brain are thought to play a part in the control of mood and the activity of the non specific arousal systems.

In order to test these possibilities we carried out a short study in the dog in which we measured c.s.f. and plasma folate activity both before, and during the administration of large oral doses of folic acid. To provide more information on the nature of the gradient in folate activity between c.s.f. and plasma we also measured the folate activity of protein-free plasma ultrafiltrate.

Many investigations within this department have shown that changes in the synthesis and release of 5-hydroxytryptamine and

dopamine are reflected by changes in the levels of their acid metabolites, 5-HIAA and HVA, in the c.s.f. (see Section 1A page 19). Therefore to test for an effect of folic acid therapy on brain amine synthesis we have examined the concentrations of 5-HIAA and HVA in the dogs' ventricular and cisternal c.s.f., before and during the treatment period.

METHODOLOGY

Animal and Experimental Procedures

As in the anticonvulsant studies the animals used were adult male Beagle dogs with permanently implanted ventricular and cisternal guide tubes. Throughout the period of the experiment the dogs were kept on a standard diet (Pedigree Chum, Petfoods Ltd.) and sampling of body fluids was carried out between 09.30 and 12.00 hours.

After a period to establish control values for the parameters being measured (folate activity in c.s.f., plasma and plasma ultrafiltrate; HVA and 5-HIAA concentrations in ventricular and cisternal c.s.f.) the three dogs (originally four, but one developed a severe infection and had to be put down) were put on a course of folic acid for a period of five weeks.

Folic acid (5 mg/day) was administered orally in tablet form, 2.5 mg at 09.00 hours and 2.5 mg at 17.00 hours. On a weight for weight basis this is equivalent to a dose of 25 mg/day in humans and as such it is nearly twice the normal

therapeutic dose (15 mg/day) administered to humans in the trial reported by Hunter et al. (1970).

The blood and c.s.f. samples were withdrawn using the techniques described in Section 1A (pages 33-35). For the preparation of the plasma ultrafiltrate a 10 ml portion of venous blood was centrifuged at 1,900 g for 10 minutes, at room temperature, and the plasma fraction was carefully removed and treated as detailed below.

Preparation of Plasma Ultrafiltrate

Plasma ultrafiltrates were prepared by a modification (Moir, 1971) of the method of McMenamy, Lund, Van Marcke and Oncley (1961). After careful washing and drying, 1 foot lengths of Visking dialysis tubing (size 8/32, Scientific Instrument Centre Ltd.) were inflated, folded in the middle, and placed inside a 12 ml centrifuge tube. The folded tubing rested on a small teflon plug (13 mm diameter, 4 mm thick, serrated at the edge) which blocked off the conical section of the centrifuge tube (Fig. 1: 13).

4 ml of plasma were then run into the two side arms of the u-tube formed by the tubing, and the centrifuge tube was tightly stoppered, jamming the top of the dialysis tubing between the side wall of the tube and the stopper. The tube was then centrifuged at 1,900 g for one hour at 37°C. The ultrafiltrate collected in the conical section of the tube, below the teflon plug. Under these conditions 4 ml of plasma

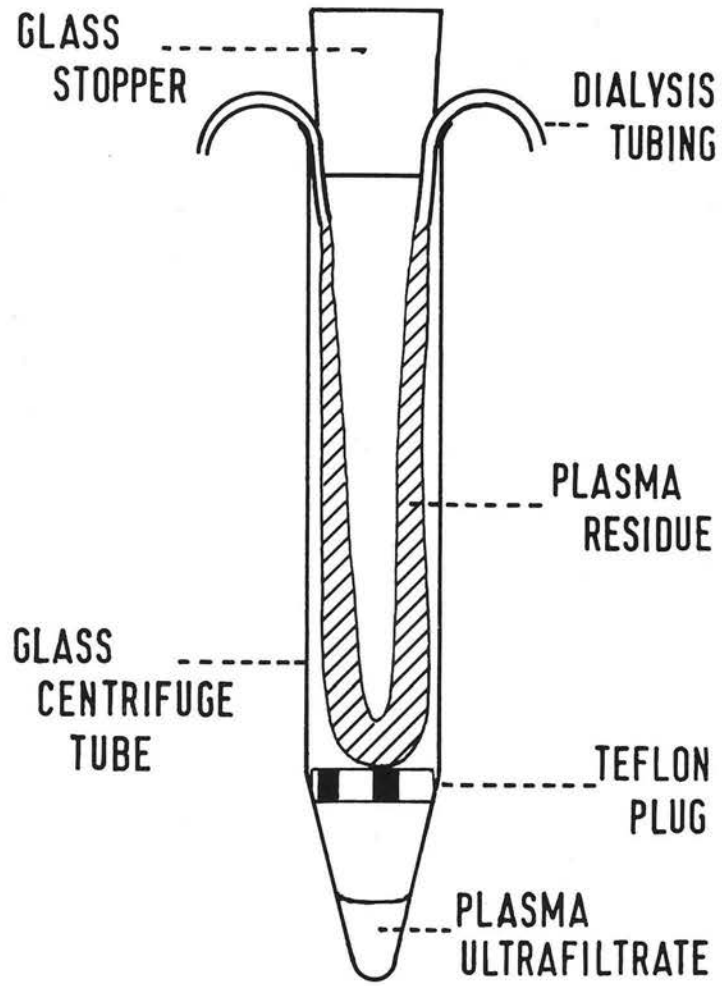


FIG. 1:13 Apparatus for preparation of plasma ultrafiltrate.

provided about 0.75 - 0.80 ml of ultrafiltrate.

For folate assay an 0.5 ml aliquot of ultrafiltrate was pipetted into a labelled glass vial containing 5 mg ascorbic acid. The vial was then stored at -20°C until assay.

BIOCHEMICAL ANALYSES

Estimation of HVA and 5-HIAA in cerebrospinal fluid

The basic methodology for HVA and 5-HIAA analyses has been described in Section 1A (pages 35-41) but this basic procedure was modified in three different ways.

Firstly, the double extraction of the acids with two 10 ml volumes of ethyl acetate was replaced by a single extraction with one 10 ml volume of butyl acetate. This modification considerably shortened the time of an assay without any decrease in the efficiency of extraction of HVA and 5-HIAA.

Secondly, for the extraction of 5-HIAA from the butyl acetate back into aqueous solution, the phosphate buffer used in earlier studies (0.1M, pH 7.4) was replaced by the borate buffer already used in the back extraction of HVA (0.1M, pH 8.6). This buffer gave a slightly lower blank in fluorimetry readings.

Finally, the Aminco-Bowman spectrophotofluorimeter used in the earlier study was replaced by a Perkin Elmer MFP-2A spectrophotofluorimeter. By the use of special micro cells available with this instrument it was possible to measure the fluorescence of 0.5 ml of solution. This facility, together with the higher

sensitivity of the instrument allowed the borate buffer extract for HVA assay to be split into two 0.5 ml fractions, one of which was used to prepare a "sample blank". One of the 0.5 ml fractions was treated, as before, with 0.5 ml 5N ammonia solution and 0.1 ml of freshly prepared ferricyanide solution. After exactly 4 minutes the reaction was terminated by the addition of 0.1 ml of freshly prepared l-cysteine solution (100 mg % w/v). The other 0.5 ml fraction was treated with the same reagents but in the reverse order, with the cysteine solution being added before the ammonia and ferricyanide solution. Both solutions were then read on the spectrophotofluorimeter and the latter fraction acted as a "sample blank" for the first fraction. This adaptation increased the sensitivity and accuracy of each HVA estimation.

Estimation of folate activity in samples of plasma, plasma ultrafiltrate and c.s.f.

The basic methodology for the folate assays was unchanged from that described in Section 1A (pages 41-47). The plasma ultrafiltrate samples, being protein free, were not subjected to a protein precipitation procedure. During the period of folic acid administration very much higher levels of folate activity were encountered in the plasma and plasma ultrafiltrate samples, and it was necessary to do a preliminary assay with different dilutions of samples, to establish what dilutions were necessary to bring the activity of the samples on to the standard curve.

RESULTS

In this study no distinction has been made between ventricular and cisternal c.s.f. when assaying for folate activity. Therefore the c.s.f. folate activities reported in this section refer, in most cases, to means of the estimates obtained from paired samples of ventricular and cisternal c.s.f. The justification for this policy was that the previous study (Section 1A, page 49) had shown very little difference between the folate activities of ventricular and cisternal c.s.f.

Pre-Treatment Control Period

C.S.F. and Plasma Folate Concentrations

At 18.3 $\pm$ 6.1 ng/ml (mean $\pm$ s.d., 28 estimates from 3 dogs) the mean c.s.f. folate concentration during the control period of this study (Table 1:12) was significantly lower ($t = 10.3$, $p < 0.001$) than the value of 41.7 $\pm$ 11.4 ng/ml (mean $\pm$ s.d., 83 estimates from 4 dogs) which was found in the control period of the previous anticonvulsant study (Section 1A). The mean control plasma folate concentration of 8.2 $\pm$ 3.8 ng/ml (mean $\pm$ s.d., 21 estimates from 3 dogs) in the present study was not significantly different from the earlier figure of 12.2 $\pm$ 5.4 ng/ml (mean $\pm$ s.d., 31 estimates from 4 dogs).

The difference in c.s.f. folate activities between the two control periods cannot be ascribed to the use of a different "population" of dogs since they were all derived from similar

TABLE 1:12 : Folate Concentrations in Plasma, Protein-Free Plasma Ultrafiltrates and C.S.F. Before and After Chronic Folic Acid Administration.

	DAY	1	5	8	16	20	22	26	29	34	36	47	54	60	64	67	71	74	81	88
	DOG																			
plasma ng/ml	A 2	13.5	8.5	6.4	9.4	13.6	4.4	14.2	4.2					108	94	46				
	A 5	17.2	5.3		5.5	6.2	9.8		4.8				117	114		45	80	104	85	
	A 6		4.5	9.6	5.3	5.3	6.5	7.1	11.1						76	44	83	50	57	
plasma ultra-filtrate ng/ml	A 2	5.3	3.5	2.8	3.3	2.8	3.8	4.3	3.8					22	19	6.8				
	A 5	4.0	2.3		3.3	4.3	3.8		2.8				23	23		11.5	14	17	18	
	A 6		3.3	3.5	3.0	3.3	2.8	3.3	3.8						15	6.5	23	8	15	
c.s.f. ng/ml	A 2	24	27	17.5	17.3	15.1	14.9	16.6	26	22	18.3	23	22	20	17.4	24	19.0	23	24	23
	A 5		13.8	7.5	9.0	10.3	14.3		18.5	19.5	15.3	22	15.5	16.5	28	14.3	18.5	14.0	15.5	15.3
	A 6	16.0	17.0	16.0	13.8		15.8	23	23			37		28	27	24	36	30	29	32
																FOLIC ACID				

breeding stock. In fact dog A2 was common to both studies. The rest period of three months between the experiments makes it unlikely that dog A2 was still being affected by the earlier anticonvulsant treatment. Nor is it likely that a change in the dietary intake of folate could be held responsible since both groups were maintained on the same standard diet. The techniques of withdrawal and preparation of c.s.f. were identical in both studies.

One possible explanation for the different c.s.f. folate activities may lie in the fact that two different preparations of medium were used in assaying the two different groups of samples. One might speculate that this produced a situation where certain factors present in the c.s.f. samples, but not in the folic acid standards, interacted differently with the two media giving two different estimates of c.s.f. folate activity despite similar standard curves for pure folic acid. Certainly we have had experience of at least one occasion where a supposedly sound batch of medium produced sensible figures for plasma folates, but nonsensical figures for c.s.f. folate.

Unfortunately such difficulties in the standardisation of microbiological assay procedures are only too common as Table 1:13 illustrates. This Table lists various "normal" values for human serum folate as measured by *L. casei* assay. As can be seen, the means alone range from 5.1 ng/ml (Temperley and Horner, 1966) to 16.0 ng/ml (Santini et al., 1966). The variation within any particular assay may be very much less. For instance Waters (1963) found a coefficient of variation of only

TABLE 1:13 : Serum folate concentrations in normal subjects

Author	Range ng/ml	Mean
Toennies et al. (1956b)	2.6 - 10.5	5.5
Herbert et al. (1960)	7.5 - 24.0	-
Cooperman et al. (1960)	4.0 - 45.6	15.9
Waters & Mollin (1961b)	5.9 - 21.0	9.9
Hansen & Weinfeld (1962)	3.7 - 9.3	5.4
Grossowicz et al. (1962)	3.2 - 15.0	8.3
Davis & Kelly (1962)	2.7 - 18.5	5.9
Spray (1964)	21. - 28.0	7.8
Kershaw & Girdwood (1964)	4.9 - 18.5	9.5
Reizenstein (1965)	2.4 - 7.4	4.6
Santini et al. (1966)	8. - 29	16.0
Grzesiukowicz et al. (1965)	6.7 - 14.6	9.2
Giles (1966)	3.4 - 11.6	6.6
Temperley & Horner (1966)	2.1 - 9.5	5.1
Vanier & Tyas (1966)	7 - 20	13.1
Spector & Hunter (1966)	3 - 11.5	7.4
Banerjee & Chatterjea (1965)	3.0 - 22.3	7.2
Magnus (1967)	3.0 - 11.0	5.7
Leevy et al. (1965)	4.0 - 10.0	-
Cooper & Lowenstein (1964)	4.0 - 18	8.1

15% when carrying out repeat assays on the same serum sample. Therefore while comparisons of folate activity may be valid within a particular series, it may be dangerous to compare values between series.

However, despite these doubts about the methodology, we cannot exclude the possibility that the observed difference in control c.s.f. folate between the two studies may not be a methodological artifact but an accurate reflection of a real change. In two recent studies involving humans (Spaans, 1971; Shaw, MacSweeney, Johnson, O'Keefe, Naidoo, Macleod, Jog, Preece and Crowley, 1971) comparable changes in c.s.f. folate activity have been reported to occur spontaneously over periods of 1-3 months.

Of the two different values which we found for control c.s.f. folate activity in the dog, the figure from the present study of 18.3 $\pm$ 6.1 ng/ml (mean $\pm$ s.d.) was very much nearer to the values of 20.9 ng/ml (Reynolds, Preece and Chanarin, 1969), 23.6 ng/ml (Wells and Casey, 1967) and 14.2 ng/ml (Weckman and Lehtovaara, 1969) which have been reported for human lumbar c.s.f.

Relationship between c.s.f. and plasma folate activities

In the previous study (Section 1A) there was evidence for a positive correlation between c.s.f. and plasma folate activities (see page 49) but in the present study there was no correlation between these two parameters in any of the three dogs (Table 1:12). These conflicting findings were particularly puzzling with respect

to dog A2 which was common to both series of experiments.

From human studies also there have been conflicting reports on the relationship between c.s.f. and plasma folate activity. Wells and Casey (1967), Reynolds et al. (1969), Jensen and Olesen (1971) and Shaw et al. (1971) all report finding a significant positive correlation between c.s.f. and plasma folate activity in paired samples withdrawn at the same time from the same individual. However, the largest survey (paired data from 416 patients), carried out by Weckman and Lehtovaara (1969) gave no evidence for such a correlation. The results of the study by Spaans (1971) are inconclusive on this point but on balance tend to agree with the findings of Weckman and Lehtovaara (1969) that there is no correlation between c.s.f. and plasma folates.

The assessment of these various studies is hindered by two problems. The first is that minor differences in sample preparation and assay technique between various laboratories may influence their results. The second problem arises from the ethical difficulties of obtaining c.s.f. from healthy control subjects. Because of these difficulties all the human studies have had to be restricted to groups of patients who were being investigated for some neurological or psychiatric complaint. The question therefore arises as to whether the same "population" was being observed in the various studies.

This topic has been fully discussed by Wells (1969).

To illustrate the complexity of the situation consider the following data from Wells and Casey (1967) (Group I) and Weckman and Lehtovaara (1969) (Group 2).

	Plasma folate (ng/ml)	C.S.F. folate (ng/ml)	C.S.F./Plasma Correlation
Group 1 (n = 30)	5.4	23.6 ± 11 (s.d.)	Positive
Group 2 (n = 416)	5.5	14.3 ± 10 (s.d.)	x

It can be seen that these two studies differ not only on the question of a c.s.f./plasma correlation with respect to folate activity, but also on the more basic issue of the c.s.f. folate concentration. One is tempted to think that these two observed differences may be related to one common difference between the two studies. As to what this difference might be one can only speculate. It is possible that two different classes of patients were being studied but this does not seem likely. More probable is some systematic difference in the time of day at which samples were withdrawn. Another possibility is that some constant error in sample preparation or assay technique was affecting the folate assays, particularly of c.s.f., either producing a c.s.f./plasma correlation when none was present, or masking the existence of a real correlation.

It seems certain therefore that many problems in sampling procedures and folate methodology will have to be resolved before the relationship between c.s.f. and plasma folate activities can be properly evaluated.

Plasma Ultrafiltrate

The plasma ultrafiltrate studies revealed that much of the folate activity in dog plasma is not freely diffusible. During the control period the plasma ultrafiltrate was found to have only 43 ± 3% (mean ± s.d.) of the folate activity of whole plasma (Table 1:12). These figures are in agreement with earlier studies on the plasma binding of folate in man (Johns, Sperti and Burgen, 1961), in the dog (Goresky, Watanabe and Johns, 1963) and in the rat (Neal and Williams, 1965). Surprisingly, during the control period, there was no correlation between the folate activities of paired samples of plasma and plasma ultrafiltrate (Table 1:12). This lack of correlation may be due to the combination of a small range of values and a relatively large error variation in the assay procedure.

HVA and 5-HIAA in C.S.F.

The HVA and 5-HIAA assays on samples of c.s.f. taken during the control period confirmed the gradient in concentration of these acids which is known to exist between the lateral

ventricle and the cisterna magna (Table 1:14). A feature of the control data was the ventricular HVA concentration in Dog A6, which was well below the "normal" for these dogs. In fact this particular dog had been newly operated on and as sometimes happens in such cases the early c.s.f. samples were markedly blood-stained. The low HVA value for this dog (Table 1:14) can be considered a direct result of this contamination since blood is known to interfere with the HVA assay. This was confirmed by repeat control estimates in the same dog, some three months later, when a ventricular HVA concentration of 1501 ± 1009 ng/ml (mean ± s.d., 16 estimates) was found.

Treatment with Folic Acid, 5 mg/day

Plasma and Plasma Ultrafiltrate

After the start of oral folic acid treatment there were striking increases in the folate activity of both plasma and plasma ultrafiltrate (Table 1:12, Fig. 1:14).

The plasma folate activity rose ten fold from 8.2 ± 3.8 ng/ml (mean ± s.d.) to 79 ± 27 ng/ml (mean ± s.d.). This change was significant in each of the three dogs ~~at a probability of less than one in a thousand~~, the probability of there being no difference being less than one in a thousand.

TABLE 1:14 : Homovanillic acid (HVA) and 5-Hydroxyindol-3-ylacetic (5-HIAA) in c.s.f. before and after chronic folic acid administration.

Sample	Dog	5-HIAA (ng/ml)		HVA (ng/ml)	
		before	after	before	after
c.s.f. from lateral ventricle	A2	297±53(9)[1]	250± 45(7)	1768±215(10)	1977±542(7)
	A5	297±43(6)	159±111(6)	1137±446(7)	1019±477(6)
	A6	131±47(5)	137± 47(5)	279±223(6)[2]	1211±670(5)
c.s.f. from cisterna magna	A2	41± 7(7)	35± 6(5)	114± 33(9)	103± 41(6)
	A5	19± 6(5)	20± 5(4)	36± 8(6)	39± 18(3)
	A6	37± 8(4)	16 & 52	99± 35(5)	80±148

[1] Mean ± standard deviation (number of samples in parentheses)

[2] Abnormally low initial values due to contamination with blood; control values taken three months after stopping folic acid administration - 1501 ± 1009 (16).

The plasma ultrafiltrate however displayed only a five fold rise in folate activity from 3.5 ± 0.7 ng/ml (mean ± s.d.) to 15.8 ± 6.0 ng/ml (mean ± s.d.). This change was also statistically significant in each of the three dogs ($p < 0.01$ for dogs A2 and A6, $p < 0.001$ for dog A5).

It should be noted that these changes represent a decrease in the proportion of folate activity which is ultrafilterable. During the control period plasma ultrafiltrate had a folate activity 43 ± 3% (mean ± s.d.), that of whole plasma. After folic acid treatment this percentage dropped to 21 ± 2% (mean ± s.d.). This finding may indicate that the increased folate activity in plasma is in a form which is more tightly bound to plasma proteins than are normal plasma folates.

During the period of folic acid treatment there was a significant correlation ($r = +0.88$, $p < 0.001$) between the folate activities of plasma and plasma ultrafiltrate.

C.S.F.

In view of the fact that c.s.f. folate activity is normally some three times greater than the plasma folate activity, in both man (Herbert and Zalusky, 1961) and dog (Table 1:3) we, in company with Hunter et al. (1970), expected that oral folic acid supplements might have an even greater effect on the c.s.f. folate activity than on the plasma folate activity.

This was not the case. A ten fold rise in plasma folate activity during the treatment period was associated with only a 22.4% increase in the folate activity of c.s.f. (Table 1:12, Fig. 1:14). From a control period mean of 18.3 ± 6.1 ng/ml (mean ± s.d.) the folate activity of c.s.f. rose to a mean of 22.4 ± 6.2 ng/ml (mean ± s.d.). Even this small increase could not be immediately attributed to the folic acid treatment since dogs A5 and A6 both showed a significant positive regression in c.s.f. folate activity throughout the control period (Fig. 1:15). Thus oral folic acid administration produced no significant increase in c.s.f. folate activity despite producing very large increases in plasma folate activity.

This inability of oral folic acid therapy to raise the folate activity of the c.s.f. has recently been confirmed by a number of similar studies in man (Spaans, 1971; Shaw et al., 1971; Hunter, Barnes, Kantamaneni and Duncan, 1971).

In the light of these results it appears unlikely that the mental changes observed by Hunter et al. (1970) were due to an increased folate activity in the central nervous system.

HVA and 5-HIAA in C.S.F.

We have found no evidence that oral folic acid treatment alters the concentration of either HVA or 5-HIAA in the ventricular or cisternal c.s.f. (Table 1:14). The very marked

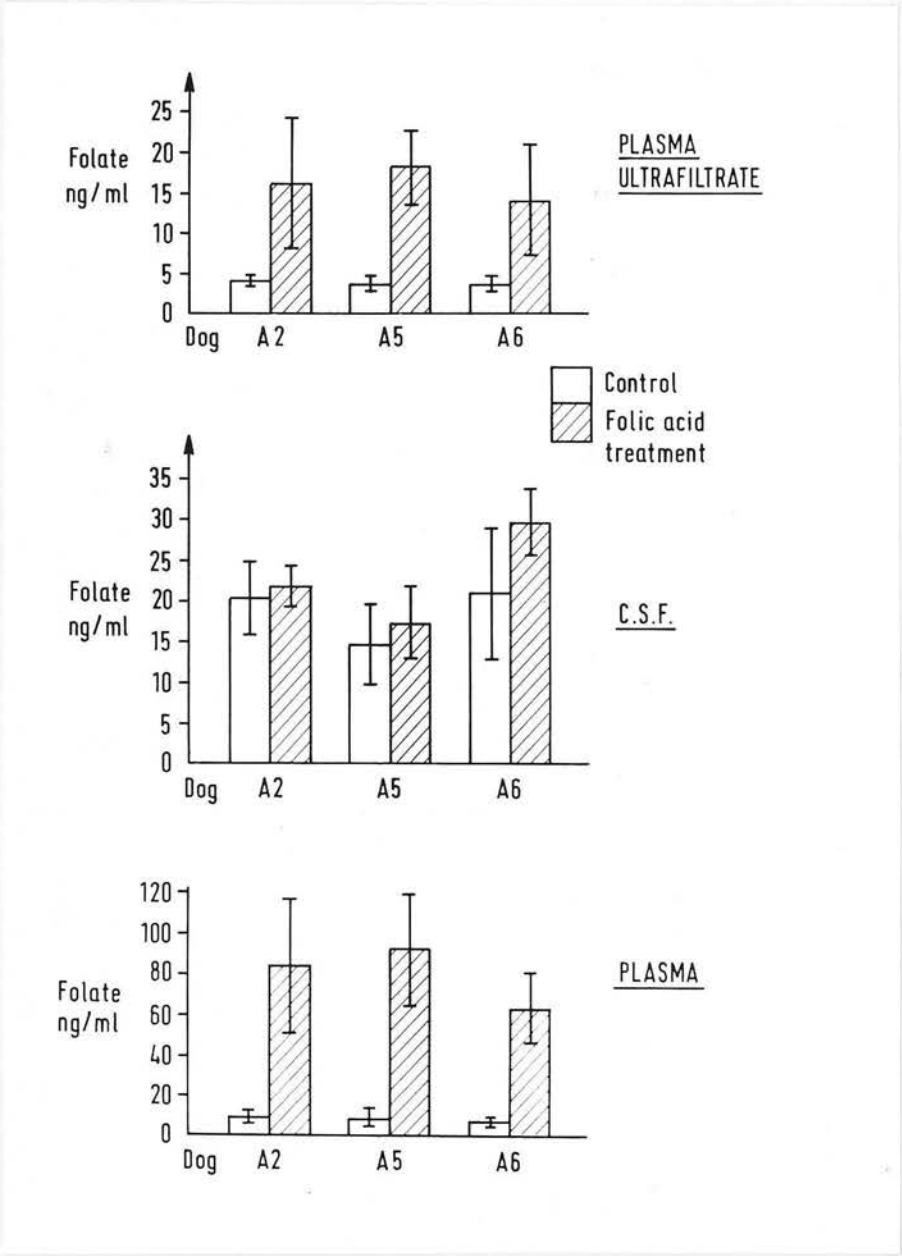


FIG. 1:14 Effect of folic acid treatment on the folate activities of plasma, plasma ultrafiltrate and c.s.f. The columns represent mean activities while the vertical bars represent standard deviations.

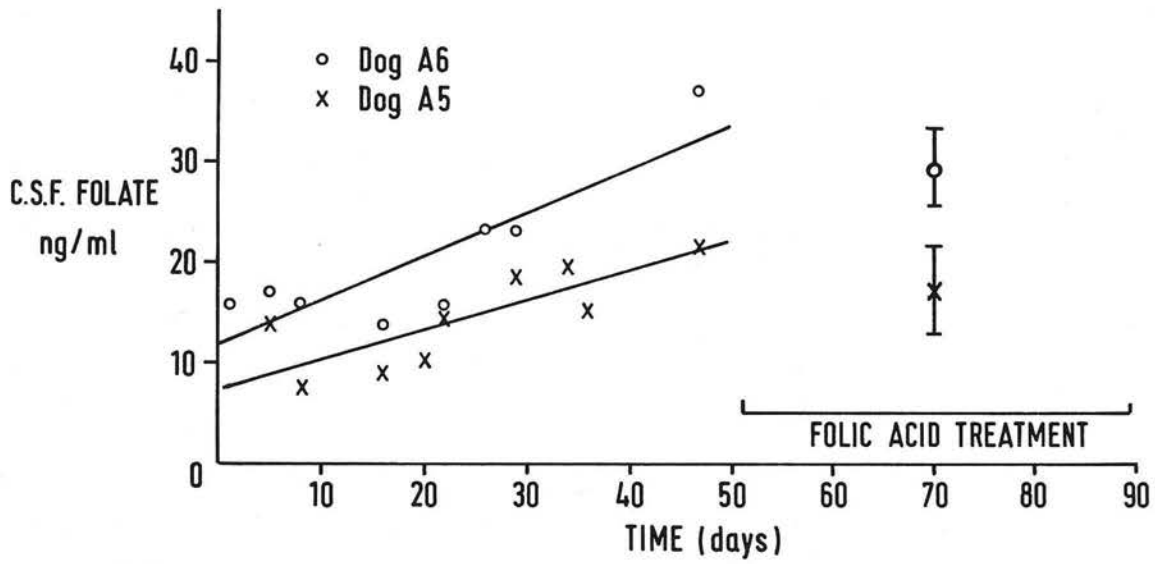


FIG. 1:15 Regression of c.s.f. folate activity in dogs A5 and A6 during the control period prior to treatment with folic acid. The points displayed during the treatment period represent the means (± standard deviations) of the c.s.f. folate activities in the two dogs during this period.

before/after treatment difference in the ventricular HVA concentration of dog A6 was caused by a methodological problem which has been discussed earlier.

From these results one can infer that oral folic acid administration has no effect on the synthesis in the brain of either dopamine or 5-hydroxytryptamine (see earlier discussion, page 66).

This is not surprising since it appears that the folate activity in the central nervous system is unaffected by large oral doses of folic acid.

These findings in the dog have recently been confirmed in man by Hunter et al (1971). When they administered folic acid orally, at a dosage of 15 mg/day (splitting the daily dose) over a period of a few weeks, they found no evidence of any change in the concentrations of folate, HVA and 5-HIAA in the lumbar c.s.f. However, 4 hours after a single large oral dose of 30 mg folic acid they found a significantly lowered HVA concentration in the lumbar c.s.f. They attributed the differing results of these two folic acid treatments to the fact that the latter treatment achieved very high plasma folate concentrations (525 $\pm$ 92 ng/ml, mean $\pm$ s.e.).

<u>The distribution of ^{14}C-methyltetrahydrofolate between c.s.f. and plasma</u>.

We have carried out two short preliminary experiments in rabbits to examine a hypothesis which might explain some of the

rather unexpected results of the folic acid studies.

Active transport of the methyltetrahydrofolate derivative into the c.s.f. has been proposed as a mechanism (Levitt et al. 1969) to account for the concentration gradient in folate activity which exists between c.s.f. and plasma (Herbert and Zalusky, 1961). The nature of the active folate derivatives in c.s.f. is not known but it is known that methyltetrahydrofolate is the predominant plasma form (Herbert et al., 1965). The lack of effect of oral folic acid treatment on c.s.f. folate activity need not rule out the active uptake of methyltetrahydrofolate into the c.s.f.: the large increases in plasma folate activity which are seen during such treatment are almost certainly due to unreduced folic acid (Butterworth et al., 1969; Melikian, Paton, Leeming and Portman-Graham, 1971), a compound which enters c.s.f. at a much slower rate than methyltetrahydrofolate (Levitt et al., 1969).

^{14}C-methyltetrahydrofolate (Radiochemical Centre, Amersham), in aqueous ascorbate solution, was injected intravenously into two rabbits. Venous blood samples were withdrawn at intervals, up to the time when the animal was bled out under pentobarbitone anaesthesia. For each blood sample taken a plasma ultrafiltrate was prepared as described on page 69 . A cisternal c.s.f. sample was withdrawn at the time of death. After death the brain was removed, weighed and homogenised in 3 volumes of water.

Aliquots of plasma, plasma ultrafiltrate, c.s.f. and brain

homogenate were taken up in a minimal volume of solubiliser (N.C.S., Searle and Co., Ltd.) and counted in a liquid scintillation counter (Mark II, Nuclear Chicago) using toluene scintillant solution (see page 214).

As Figs. 1:16 and 1:17 indicate there was little evidence to suggest that methyltetrahydrofolate is actively concentrated by the c.s.f. Both at 3.2 and 5.0 hours after the injection of ^{14}C-methyltetrahydrofolate the plasma was more highly labelled than the c.s.f. If ^{14}C-methyltetrahydrofolate was being actively transported into the c.s.f. it must have been very rapidly removed by uptake into the brain. In both animals the brain was about 6 times more highly labelled than the c.s.f. at the time of death. Preliminary results from comparable studies in the rat suggest similar findings.

There have been two very short reports in the literature on the transport of folates between plasma and c.s.f. Levitt et al. (1969) found that methyltetrahydrofolate and formyltetrahydrofolate, both natural folate derivatives enter dog c.s.f. much more readily than do folic acid and methotrexate. Woodbury and Kemp (1971) state that ^{3}H- folic acid is concentrated in the c.s.f. one hour after administration. Unfortunately they give no details as to the species of animal being used, nor the dosage or route of administration of the folic acid. Until the experimental details of both these studies are known it will be difficult to compare their findings with our own. Certainly our own data do not support the hypothesis (Levitt et al., 1969) that the

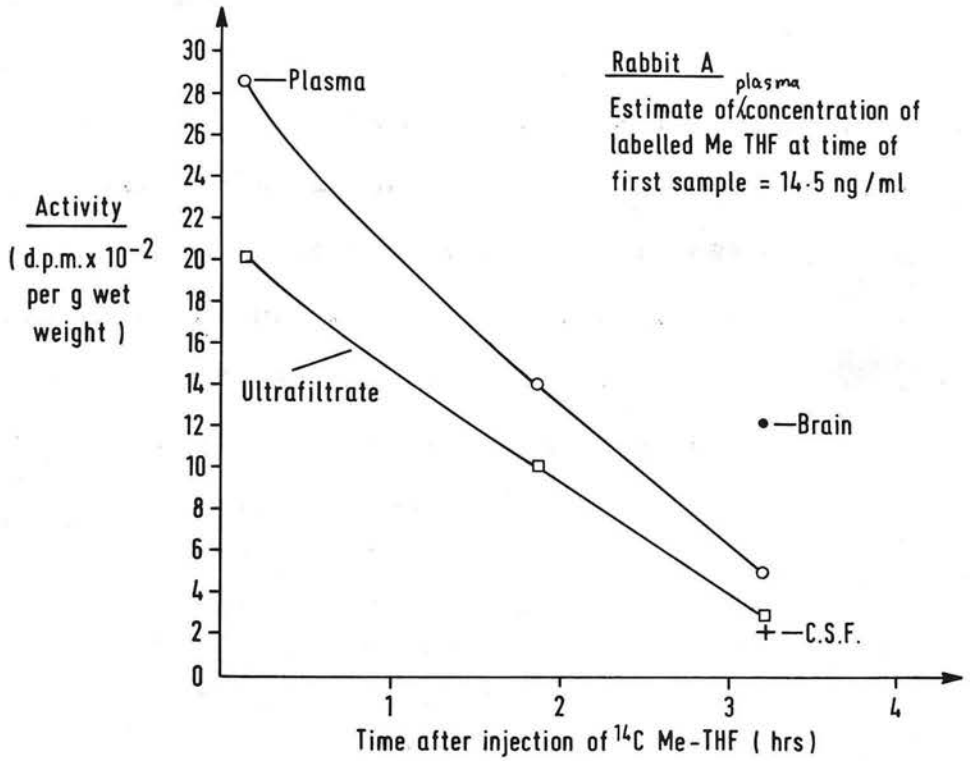


FIG. 1:16 Distribution of ^{14}C activity in plasma, plasma ultrafiltrate and brain of rabbit A after the intravenous injection of ^{14}C-Methyltetrahydrofolate (^{14}C - MeTHF).

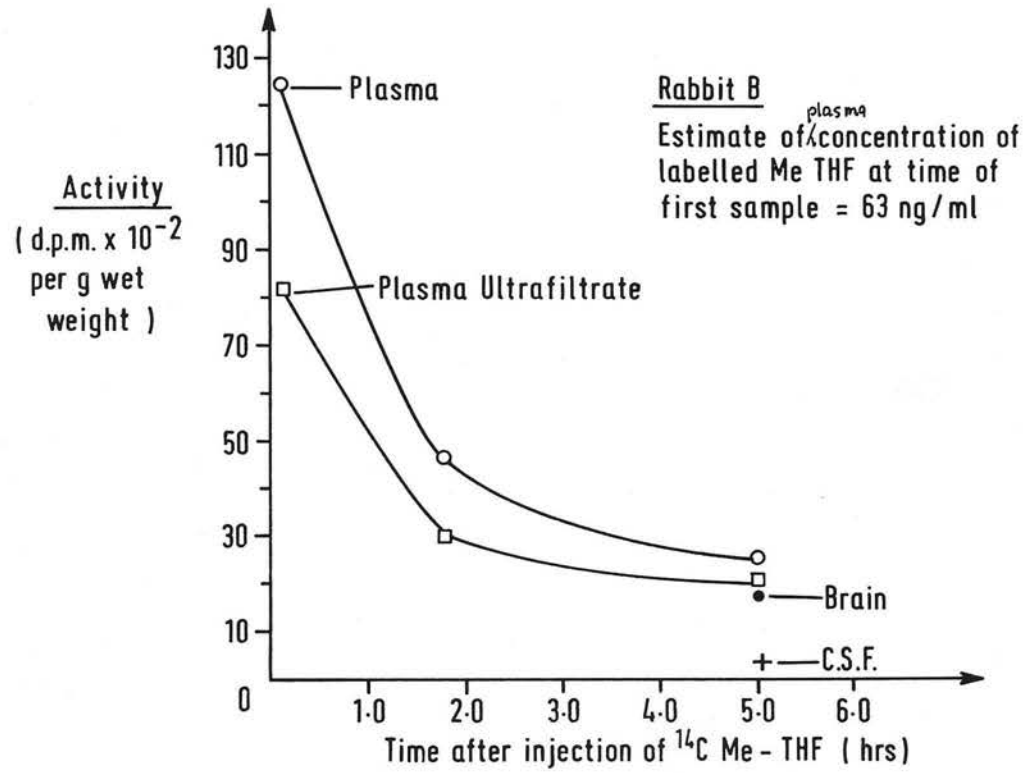


FIG. 1:17 Distribution of ^{14}C activity in plasma, plasma ultrafiltrate and brain of rabbit B after the intravenous injection of ^{14}C-methyltetrahydrofolate (^{14}C - MeTHF).

active transport of methyltetrahydrofolate into the c.s.f. accounts for the concentration gradient in folate activity between c.s.f. and plasma.

DISCUSSION

The finding that oral folic acid treatment can raise the plasma folate activity to very high levels without any appreciable effect on the folate activity of c.s.f., has considerable bearing on a number of issues in the field of folate metabolism.

Firstly, if, as has been suggested (Wells and Casey, 1967; Hunter et al., 1970), the folate activity of c.s.f. reflects the folate content of the brain one might infer from our findings that oralfolic acid therapy has little effect on the folate content of the brain. It is thus unlikely that the mental changes observed by Hunter et al., (1970) following administration of folic acid to healthy volunteers could be, as they suggest, due to toxic effects of folate on the central nervous system. Certainly we have found no evidence that oral folic acid, as administered by Hunter et al. (1970), has any effect on the turnover of dopamine and 5-hydroxytryptamine in the central nervous system.

The second issue on which our present results have some bearing is the relationship between folic acid, anticonvulsants and epilepsy. Reynolds (1967) has reported that folic acid treatment in epileptics on anticonvulsants causes an improvement in their mood and an increase in drive. Fit frequency and

severity was also increased (see review - Reynolds, 1971). Folic acid therapy has also been said to bring about a significant improvement in patients with organic psychoses, endogenous depression and schizophrenia (Carney and Sheffield, 1970). In the light of our present findings it is doubtful whether a specifically central action of folic acid can be responsible for these effects of folic acid therapy. Indirectly therefore, our findings lend support to the hypothesis that the effect of folic acid on fit frequency and severity is principally a result of increased peripheral metabolism of the anticonvulsant drug, leading to sub-therapeutic anticonvulsant drug levels. (Jensen and Olesen 1970; Baylis, Crawley, Preece, Sylvester and Marks, 1971).

The most basic issue upon which our findings have a bearing is the whole question of the transport and distribution of folates between the plasma and the c.s.f. The current state of ignorance in the field stems largely from the fact that there are no sensitive and specific assay procedures which can differentiate quantitively between the various folate derivatives (see introduction to Section 1). Thus, folate activity, as measured in the l.caseii assay, can refer to many different folate derivatives (Table 1:1) all of which may have different transport characteristics.

In normal untreated animals methyltetrahydrofolate appears to be responsible for most of the folate activity of plasma

(Herbert et al., 1965). However the question of which derivatives are responsible for the increased plasma folate activity observed after oral folic acid, is still a matter of some debate. Baker, Frank, Feingold, Ziffer, Gellene, Leevy and Sobotka (1965) reported that an oral dose of 5 mg of folic acid in man raised the l.caseii activity of plasma but had little effect on the s.faecalis activity. The authors deduced from this that methyl-tetrahydrofolate was responsible for the large increases in the plasma folate activity seen after this dose of oral folic acid. This interpretation fits well with the report that dihydrofolic acid and tetrahydrofolic acid are methylated during absorption from the gut (Chanarin and Perry, 1969). However recent studies combining microbiological assay with radioactive (Butterworth et al. 1969) and chromatographic techniques (Melikian, Paton, Leeming and Portman Graham, 1971) indicate that the increased plasma folate activity after large oral doses of folic acid is due to folic acid itself.

From our own studies with plasma ultrafiltrates it appears that oral folic acid treatment increases the proportion of total plasma folate activity which is bound to plasma proteins. This is the opposite of what one might expect on the basis of simple saturation of protein binding. One explanation may be that the change in binding is reflecting the presence of a different form of folate: one which has a higher affinity for protein binding sites than the normal plasma forms. One might

speculate that this difference in protein binding of the folate activity results from the presence of large amounts of folic acid in plasma.

On the balance of the evidence therefore, it seems likely that oral folic acid treatment leads to a high plasma concentration of folic acid itself. In normal circumstances this compound would not be present in the body: in some respects therefore oral folic acid treatment creates a "non-physiological" increase in the folate activity of plasma.

Blair (1970) has suggested that these very high tissue levels of folic acid may actually decrease the levels of the metabolically active folate derivatives. This proposal was made on the ground that unreduced folic acid may act as a competitive inhibitor of the enzyme dihydrofolic acid reductase (Bertino, Perkins and Johns, 1965). This enzyme has an important role in recycling dihydrofolate (an important side product of thymidilate synthetase) back into the general pool of metabolically active folate derivatives such as methyltetrahydrofolate and tetrahydrofolate. However the evidence from "in vivo" studies (Auletta, Mead and Waravdekar, 1970) suggests that this effect of folic acid may not be significant at normal dosage levels. Even if it were true that folic acid was inhibiting dihydrofolic acid reductase, it is doubtful whether such an action (Kaufman, 1963) would influence the availability of reduced pteridine cofactor (see earlier discussion, page 66) in the way Hunter et al. (1971) have suggested.

The interpretation that the increased plasma folate activity observed in our dogs was due to folic acid itself, suggests a simple hypothesis which might explain why c.s.f. folate activity is not raised. Levitt et al. (1969) have reported that folic acid enters c.s.f. at a very much slower rate than does methyltetrahydrofolate. Since this latter compound is the normal plasma form, selective uptake of methyltetrahydrofolate by the c.s.f. and exclusion of folic acid itself could explain both the normal c.s.f./plasma folate distribution and the altered distribution seen after oral folic acid treatment. Unfortunately, however, Woodbury and Kemp (1971) report that folic acid is selectively concentrated by the c.s.f. while we ourselves could find no evidence that methyltetrahydrofolate is selectively concentrated by the c.s.f.

Another possible explanation for the observed folate activity distributions, before and after folic acid treatment, is that folates are unable to diffuse across the choroid plexus epithelial barrier, but that they are taken into the c.s.f. by a specific transport mechanism, which is normally working at saturation level. This would explain the lack of correlation between c.s.f. and plasma folates (Weckman and Lehtovaara, 1969). By the same token, of course, such a mechanism would be highly unlikely if the reported correlations between c.s.f. and plasma folate activity (Wells and Casey, 1967; Reynolds et al., 1969; Shaw et al., 1971) proved to be correct. Perhaps the strongest evidence for such a mechanism comes from Lanskowsky's report (1970) on a case of

folate malabsorption. In order to achieve detectable plasma folate activities (in this patient) by way of oral administration, it was necessary to give very high doses of folic acid (40 mg/day). However even after the plasma folate activity was raised, either by this means or by intramuscular injection, no folate activity could be detected in the c.s.f. These findings imply that the transport of folate both from gut to plasma and from plasma to c.s.f. involve essentially similar active processes in which diffusion plays little part.

Investigation of folate absorption from the gut has established that polyglutamate forms of folate, which make up the bulk of dietary folate, must be degraded to simpler folates before they can be absorbed (Streiff and Rosenberg, 1967; Rosenberg, Streiff, Godwin and Castle, 1969; Hoffbrand, Necheles, Moldonado, Horta and Santini, 1969 ; Butterworth, Baugh and Krumdieck, 1969; Bernstein, Gutstein, Weiner and Efron, 1970). It has also been established that an active transport mechanism located in the duodenum and jejunum (Burgen and Goldberg, 1962; Cohen, 1965; Booth, 1968; Hepner, Booth, Cowan, Hoffbrand and Mollin, 1968; Hepner, 1969) could account for the rapid, and nearly complete, absorption of physiological doses of pure folic acid (Girdwood, 1953; Anderson, Belcher, Chanarin and Mollin, 1960).

Despite the proven existence of these folate transport mechanisms in the intestine it may be that the folate activity in c.s.f. is functionally isolated from the folate activity in plasma

and reflects only the folate activity of brain (Spaans, 1972). Certainly, the brain seems to be a reservoir of considerable folate activity (150 ng/mg wet weight), much of which is in a non-conjugated form (McClain and Bridgers, 1970).

In the final analysis however this hypothesis is only speculation, as indeed are all the others which we have advanced to explain the known facts on the distribution of folate activity between c.s.f. and plasma. Complete understanding of this problem will depend to a large extent on the development of reliable techniques for the separation and analysis of each individual folate derivative.

SUMMARY

SECTION 1A

Four anticonvulsant drugs, diphenylhydantoin, phenobarbitone, sulthiame and carbamazepine were administered to dogs over a period of five weeks. None of these drugs had any effect on the level of folate activity in the c.s.f. or plasma. Nor did they alter the levels of the amine metabolites, homovanillic acid (HVA) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the ventricular and cisternal c.s.f. These results are discussed in relation to the lowered plasma and c.s.f. folate activities produced in man by chronic anticonvulsant treatment.

SECTION 1B

Folic acid was administered to dogs over a period of five weeks. This treatment was found to cause a 10-fold rise in plasma folate activity and a 5-fold rise in plasma ultrafiltrate activity but to have no effect on c.s.f. folate activity. The c.s.f. levels of the amine metabolites, HVA and 5-HIAA, were also unaffected by the folic acid treatment. It was concluded that folic acid treatment would be unlikely to have any significant effect on normal cerebral function. A short ancillary study in the rabbit found no evidence for the active transport of methyltetrahydrofolate (the predominant plasma form of folate) into the c.s.f. The implications of these findings are discussed, firstly in relation to the various clinical actions which have been attributed to folic acid therapy, and secondly in relation to the normal distribution of folate activity between c.s.f. and plasma.

SECTION II

THE EFFECTS OF ANAESTHETIC AND ANTICONVULSANT DRUGS ON C.S.F. POTASSIUM FLUXES.

INTRODUCTION

Potassium and Convulsant Activity in the Brain

In 1899 Meltzer reported that intracerebral injections of potassium chlorate produced hyperactive behaviour followed by convulsions, and finally coma. Sodium chlorate injected in equimolar quantities produced no significant effects. Similar excitatory effects of potassium on the central nervous system have since been reported by many others, including Koenigstein (1951); Feldberg and Sherwood (1957); John, Tschirgi and Wenzel (1958) and Nakajima (1964).

The effects of excess potassium on the neurones in the hippocampus of the cat have been examined in detail by Zuckerman and Glaser (1968). In their experiments the inferior horn of a lateral ventricle of a conscious cat was perfused with artificial c.s.f. solutions in which varying amounts of sodium had been replaced by potassium. When the concentration of potassium in the perfusion fluid was increased to twice normal (i.e. 2.7 meq/l→ 5.4 meq/l) single shock stimuli to the dorsal hippocampus were observed to give rise to localised epileptiform discharges: a phenomenon which was never seen when the perfusion fluid contained normal amounts of potassium. Perfusion of solutions of between three and five times the normal potassium concentration produced, after a latent period, spontaneous paroxysmal discharges in the region of the dorsal hippocampus. The incidence and severity of the epileptiform activity was proportional to the increase in

the potassium concentration of the perfusion fluid. Zuckerman and Glaser therefore concluded that the activity resulted from an increased potassium concentration in the extracellular fluid of the hippocampus.

Their hypothesis assumes that potassium can pass freely from the c.s.f., across the ependyma, into the extracellular fluid of the brain. This assumption would appear to be well justified. In the first instance Wallace and Brodie (1939 and 1940) have demonstrated that iodide, bromide and thiocyanate, after introduction into the plasma, distribute in the brain extracellular fluid and the c.s.f. to an equal extent. More recently it has been shown that even large water soluble molecules such as inulin (Rall, Oppelt and Patlak, 1962; review - Rall, 1968) and ferritin (Brightman, 1965) can slowly diffuse out of the ventricular c.s.f. into the intercellular spaces of the brain. When it has been possible to compare brain extracellular fluid with c.s.f. these fluids have been found to be similar with respect to hydrogen ion and bicarbonate concentrations (Pappenheimer, 1967), and also with respect to potassium concentration (Cohen, Gerschenfeld and Kuffler, 1968). However perhaps the most direct evidence has come from studies in which the cerebroventricular system has been perfused with ^{42}K solutions containing various concentrations of potassium (Cserr, 1965; Bradbury and Davson, 1965; Katzman, Graziani, Kaplan and Escriva, 1965). It would appear from these studies that there

is a large and probably diffusion-limited exchange of potassium across the ependymal barrier.

In view of these and other experimental findings it seems likely that the effects observed by Zuckerman and Glaser (1968) were indeed due to an increased potassium concentration in the extracellular fluid of the hippocampal region. Certainly the depolarising action of potassium and its potentiating effects on transmitter release in peripheral cholinergic neuronal systems (Gage and Quastel, 1965; Parsons, Hoffman and Feigen, 1965) suggest an appropriate scientific basis for the results observed by Zuckerman and Glaser (1968). With regard to the depolarising action of potassium it is interesting that a profound depolarisation shift has been a common finding in intracellular recordings from neurones involved in experimentally induced seizure phenomena (Kandel and Spencer, 1961; Sawa Maruyama and Kaji, 1963; Matsumoto and Ajmone-Marsan, 1964; Sawa, Kaji and Usuki, 1965; Creutzfeldt, Watanabe and Lux, 1966; Sawa, Nakamura and Naito, 1968; Prince, 1968).

Considerations such as these outlined above lead one to examine the factors involved in maintaining the "status quo" of brain and c.s.f. potassium. Under normal circumstances the c.s.f. potassium concentration is very much lower than that of a plasma dialysate (Flexner, 1934), and even chronic elevation of plasma potassium has been shown by Bekaert and Demeester (1951a); Dunker (1957); Kemeny, Boldizar and Pethes (1964); Bradbury and

Davson (1965); and Bradbury and Kleeman (1967) to have very little effect on c.s.f. potassium. Again there is only a very small drop in c.s.f. potassium when the plasma potassium is chronically lowered by glucose and insulin injections (Bekaert and Demeester, 1951b). Similarily, Cserr and Rall (1967) and Cohen et al. (1967) have reported the homeostatic control of c.s.f. potassium in the dogfish and necturus maculosus respectively. Total brain potassium is also amazingly constant in the face of chronic elevation of the plasma potassium (Davenport, 1949; Leiderman and Katzman, 1953; Timiras, Woodbury and Goodman, 1954).

This finding of stable c.s.f. and brain potassium concentrations and the effects seen when this stability is experimentally disturbed suggest that a breakdown of mechanisms controlling potassium concentration in the brain and c.s.f. might play a part in the epileptic process either in its genesis, propogation or continuation. This was first put forward as a hypothesis by Green in his review on hippocampal function (1964) and it has been discussed further by Zuckerman and Glaser (1968). Fertziger and Ranck (1970) have even proposed a model in which potassium accumulation in the intestitial spaces of the brain is an important step in seizure generation.

We were attracted by this hypothesis and decided to test it further by examining whether those drugs which prevent or terminate epileptic activity, namely the anticonvulsants, have

any uniform effects on potassium dynamics in the brain and c.s.f. Woodbury and Kemp (review -1971) have talked of anticonvulsants as membrane stabilisers and have reported that diphenylhydantoin affects sodium levels in the brain and potassium levels in the c.s.f. The permeability of the "blood-brain barrier" has been reported to be increased by a variety of convulsive agents (Ovcharov, 1967), and anticonvulsants are said to block the increased permeability. As Ovcharov states, however, it is impossible to say whether the effects on permeability are primary or secondary to the convulsions.

This problem of "cause and effect" is very common in studies of model systems of epilepsy and, to avoid it, we decided to examine whether anticonvulsant drugs had any effect on the c.s.f. potassium transport mechanisms of the normal dog. Although one might argue such a study has little to do with anticonvulsant actions in epilepsy it may not be far removed since it is very likely that anticonvulsants act principally on normal brain tissue, by modifying the response to the abnormal activity of an epileptogenic focus.

The drugs included in the study were sodium thiopentone, sodium pentobarbitone, paraldehyde, diazepam and diphenylhydantoin. The first two of these are rather non-specific depressants of neuronal activity and the latter three are more specifically anticonvulsants. These particular drugs were chosen because their clinical effectiveness in status epilepticus indicated a

rapid onset of action after acute administration, thus making them suitable for use in acute experiments.

Ideally we would have liked to be able to measure the potassium concentrations of the intra- and extracellular compartments of brain during these studies but this is not yet possible in mammalian brain. Woodbury (1955) has calculated intracellular potassium in brain with the assumptions that the extracellular fluid volume is equivalent to the chloride space and that all the potassium in brain is intracellular but neither of these assumptions is valid enough to make the method reliable.

In the absence of direct methods of studying the effects of anticonvulsants on brain potassium it was decided to approach the problem by examining the transport of potassium into and out of the cerebrospinal fluid. All previous studies of this nature have been carried out by the technique of ventriculo-cisternal perfusion (Pappenheimer, Heisey, Jordan and Downer, 1962; Cserr and Pappenheimer, 1964; Cserr 1965; Bradbury and Davson 1965; Katzman et al., 1965) and it was therefore decided to adapt the "recirculatory" perfusion systemdeveloped in this department (Ashcroft, Dow and Moir, 1968) to a system which would permit "open" perfusion studies in the conscious dog. Stress should be laid on the desirability of studying the conscious animal since studies of cerebral mechanisms carried out under anaesthesia are certainly not studies of "normal" mechanisms. In this respect our studies supplement the earlier

studies of Cserr (1965), Bradbury and Davson (1965) and Katzman et al. (1965) where only anaesthetised animals were used.

Ventricular-Cisternal Perfusion

The first description of a ventricular-cisternal perfusion was by Adam, McKail, Obrador and Wilson (1938) from the Department of Pharmacology and Surgery of this University. The technique has since been widely applied to the study of the central nervous system and its environment.

In early experiments the method was used to study the release of transmitters from the brain (Adam et al., 1938), to study the response to alteration of the ionic environment of the brain (Leusen 1950, 1954 a, b), and to administer drugs directly to the ventricular surface of the brain (Royer, 1950). This qualitative type of application has continued to be most productive and has been developed (e.g. Bhattycharya and Feldberg, 1958 a, b ; Carmichael, Feldberg and Fleischauer, 1964) to the extent that it is now possible to localise the site of action of particular treatment (e.g. Zuckerman and Glaser, 1968), or the site of release of a specific compound (e.g. Beleslin, Carmichael, and Feldberg, 1964; Portig, Sharman and Vogt, 1968) to a definite area of brain.

The other major development in perfusion technique has been the introduction of analytical techniques which enable one to carry out quantitative studies on the rates of entry and/or removal of substances from the c.s.f. The pioneer work in this

field was carried out by two research groups led by Pappenheimer and Davson. In their initial study Pappenheimer and his colleagues (Pappenheimer, Heisey and Jordan, 1961) reported the ventriculo-cisternal perfusion of the conscious goat and demonstrated that diodrast and phenelsulphonpthalein were actively transported from the c.s.f. In a further paper (Pappenheimer, Heisey, Jordan and Downer, 1962) they described the perfusion technique in detail, and reported calculated rates of removal of inulin, creatinine, fructose, urea, sodium and potassium from the system. It was noted that potassium and urea appeared to be distributed in a larger volume than the other substances, and it was suggested that this might indicate that the ependymal cells lining the ventricles were more permeable to these substances. In a companion paper (Heisey, Held and Pappenheimer, 1962) they reported the detailed kinetic analysis of the perfusion system, describing how inulin dilution could be used to measure the rate of formation of c.s.f. within the ventricles. They also described the use of inulin clearance as a measure of the rate of loss of fluid from the system by bulk flow to the subarachnoid spaces.

This use of inulin dilution to measure c.s.f. formation rates and bulk absorption rates for c.s.f. has been a common feature in many studies and is worth considering in detail. The method relies on the assumption that the diffusional losses of inulin in passage through the system are negligible. The justification for this assumption which was put forward by

Heisey et al. (1962) was that the rate of removal of inulin from the system varied linearly with changes in c.s.f. pressure, almost no inulin being lost when the pressure was reduced to zero. This finding was confirmed by Bering and Sato (1963). Davson, Kleeman and Levin (1962) and Pollay and Davson (1963) both reported that the values of c.s.f. formation rate obtained from inulin dilution calculations were in good agreement with these obtained from straightforward measurements of c.s.f. flow from a cisternal cannula. In view of these findings inulin has been added to the perfusion fluid in all our studies as a marker, allowing calculation of the rates of formation and bulk absorption of c.s.f.

With the use of inulin as a "non-diffusible" marker to monitor the bulk flow of perfusion fluid, it became possible, for the first time, to carry out quantitative studies of the efflux of a substance from the c.s.f. Among the compounds where transport from the c.s.f. has been studied in this way are iodide, paraaminohippuric acid and thiocyanate (Pollay and Davson, 1963), homovanillic acid and 5-hydroxyindol-3-ylacetic acid (Ashcroft, Dow and Moir, 1968) and tryptophan (Geddes and Moir, 1969).

Of particular relevance to our own project are the studies reported in 1965 from three independent groups of workers following up Pappenheimer's original observations on potassium transport from the c.s.f. (Pappenheimer, Heisey and Jordan, 1962).

These reports came from Cserr (1965), who had given a preliminary communication (Cserr and Pappenheimer, 1964), Bradbury and Davson (1965) and Katzman et al. (1965). The studies were carried out on dogs, rabbits and cats respectively, all in the anaesthetised state.

Despite the species differences the findings were essentially the same in all the studies, namely: that the "clearance" of ^{42}K from the perfusion system was very much greater than the "clearance" of inulin; that most of the ^{42}K cleared from the ventricular spaces could be recovered from the brain (assuming that the normal c.s.f. concentration of potassium, i.e. approximately 2.9 meq/l, was adhered to in perfusion) at the end of a perfusion; that ouabain reduced the ependymal efflux of ^{42}K to roughly one quarter of its pre-ouabain level; that for influx concentrations between 0 and 10 meq/l a constant fraction of the potassium in the c.s.f. appeared to cross the ependymal or choroidal epithelial barriers; and that the influx of potassium into the c.s.f. seemed to be principally derived from brain.

These findings suggested that an examination of the effects of anaesthetics and anticonvulsants on the c.s.f. potassium fluxes might contribute to a better understanding of the relationship between potassium and convulsant activity in the brain. In view of the fact that Pappenheimer, Fencl, Heisey and Held (1965) in repeating earlier work of Leusen (1954 a, b) found that the changes in respiration seen after altering the ionic content of

c.s.f. were very much greater in the conscious animal than in the anaesthetised animal, we decided there would be major advantages in carrying out our study on conscious animals.

METHODOLOGY

Perfusion Technique

The perfusion technique used in this study was a hybrid of the open perfusion system developed by Pappenheimer's group (Pappenheimer, Heisey and Jordan, 1961) and the recirculatory perfusion system developed in this department (Ashcroft, Dow and Moir, 1968). An "open" system, that is one in which fluid is pumped from a reservoir to the lateral ventricle and collected from the cisterna magna, was chosen in preference to a recirculatory system because an open system is much simpler to analyse kinetically and reaches a "steady state" more rapidly. However, in order to carry out open perfusions in conscious free-moving animals it was necessary to find an alternative to a simple pressure-controlled outflow at the cisternal site.

This problem was solved by putting another pump on the outflow side, functioning at the same rate as the inflow pump. The pressure control within the c.s.f. perfusion system (actually the volume of fluid held between the two pumps) was thus left to the dogs' normal mechanisms for regulating c.s.f. pressure. In this respect the system is similar to a closed recirculatory perfusion.

The basic animal methodology, that is, the use of permanently implanted guide tubes to the ventricles and the cisterna magna, was identical to that described in Section I of this thesis and was unchanged from the form originally developed for recirculatory

perfusion studies (Ashcroft, Dow and Moir, 1968).

Needles

The needles used to make the percutaneous puncture and connect the external perfusion circuit to the dog's ventricular system were 21 gauge, luer-fitting stainless steel needles with a short bevel and a small side hole near the tip. A set of three such needles, one for each of the two ventricular guide tubes and one for the cisternal guide tube, was prepared to the exact length specifications (see page 28) for each dog used in perfusion experiments. By this system it was possible to ensure rapid and reproducible entry to the ventricular and cisternal spaces simply by carefully advancing the needle down the guide tube to its full depth.

Perfusion Circuit

The open perfusion system which we have employed in these experiments is shown in the photograph, Fig. 2:1. Fig. 2:2 is a diagrammatic representation of the same system. As can be seen in Fig. 2:2 the basic features of the system are a reservoir of artificial c.s.f., two pumps, both with identical flow rates, the dog itself and a fraction collector to collect successive aliquots of the perfusate.

These component parts of the system were linked up with silicone rubber tubing of 0.8 mm internal diameter and 4.0 mm external diameter (Watson-Marlow). Luer-lock adapters (Fig. 1:7)

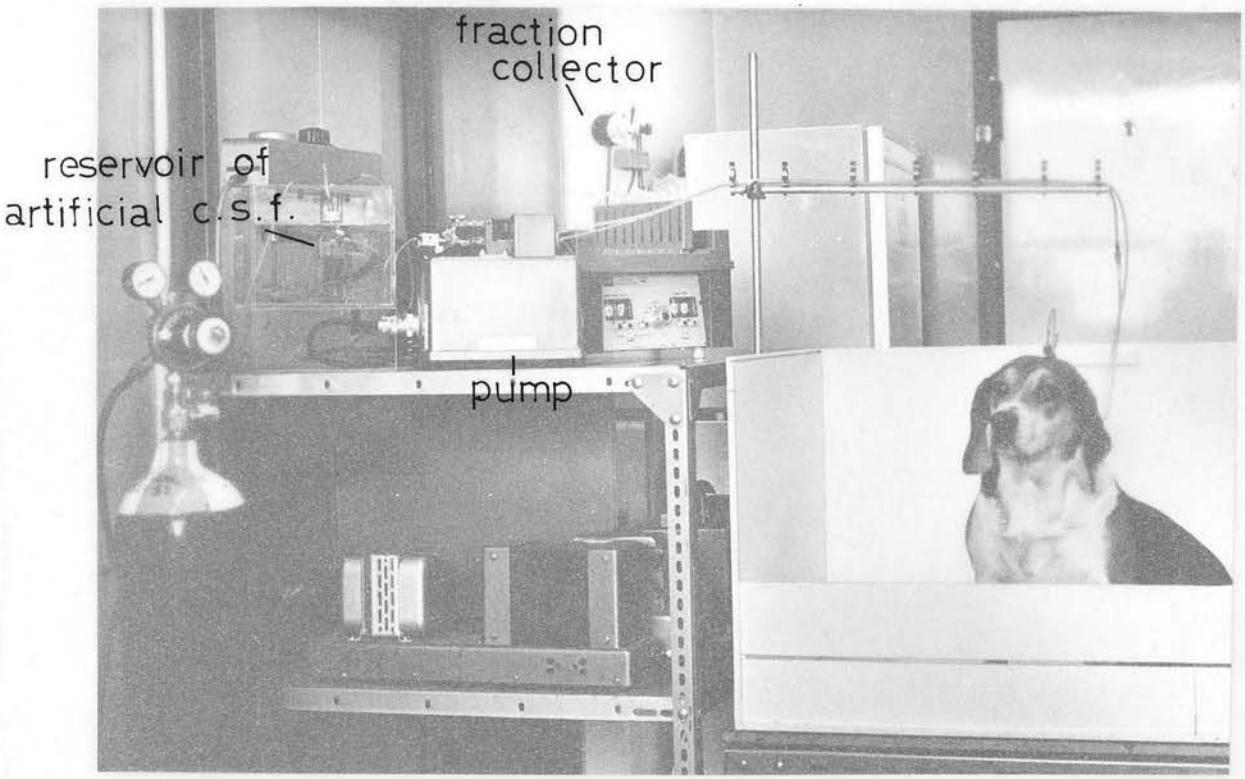


FIG. 2:1 Photograph of perfusion system showing a dog being perfused.

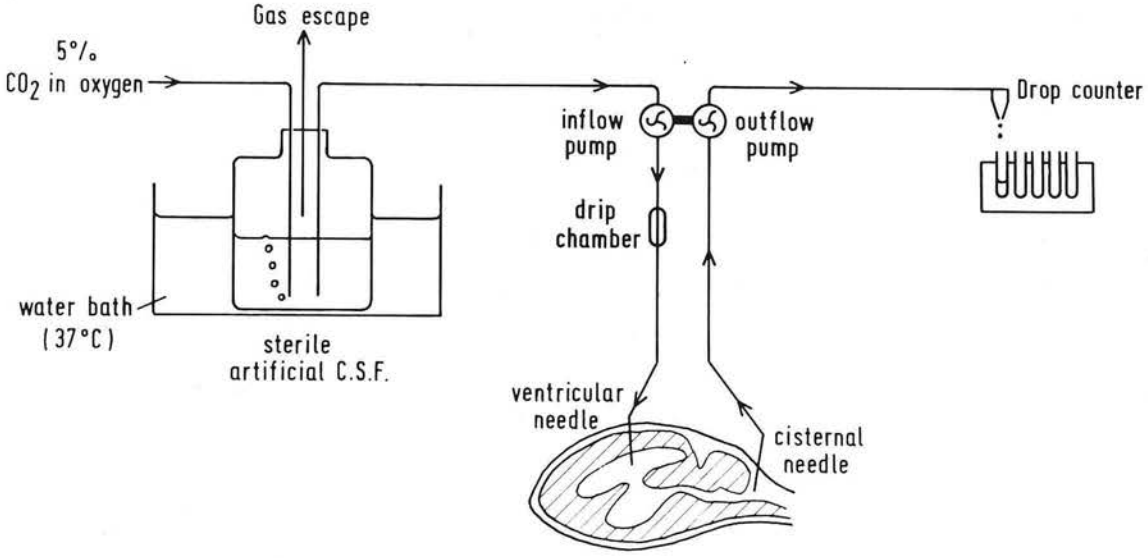


FIG. 2:2 Diagram of "open" cerebroventricular perfusion system.

The inflow and outflow pumps are separate modules of the same multichannel pump. Both modules are set to pump at an identical flow rate.

were used to connect the tubing of the external circuit to the perfusion needles in the ventricular and cisternal guide tubes.

Inflow Side of Circuit

The inflow perfusion fluid (page116)was contained within a reservoir bottle kept at 37°C by a heated water bath. The rubber cap of the bottle was pierced by three hollow needles, one to bubble 5% CO_2 in oxygen into the fluid, one to withdraw fluid and one to allow the injected gases to escape. From this heated reservoir the artificial c.s.f. was pumped to the inflow needle, leading into one of the dog's lateral ventricles. Inserted into the inflow tubing, between the pump and the inflow cannula, was a small glass drip-chamber which allowed rapid continuous assessment of the rate of inflow into the lateral ventricle.

Outflow Side of Circuit

From the needle in the cisternal guide tube outflow fluid was pumped directly to a fraction collector. The fraction collector was set to change sample tubes after a fixed number of drops, this number depending on the volume of fluid required for assay procedures and on the number of sampling points required in a fixed time interval.

Pumps

In early perfusion experiments an M.H.R.E. Flow Inducer pump (Watson Marlow Ltd.) was used, with two of the available channels

acting as inflow and outflow pumps. Before each perfusion the two channels were checked and, if necessary, adjusted to ensure that both were pumping at 0.300 ± 0.003 ml/min.

In later experiments this pump was unavailable and a Technicon fractionating pump was used. This pump, while perhaps delivering a more constant flow over a period of several hours, suffered from the disadvantage that it was capable of exerting considerable pressure should any block develop in the perfusion circuit.

As is shown in the results section, both pumps were relatively non-pulsatile and did not cause any serious disturbance in the pressure of c.s.f. within the dog's ventricular system.

Preparation of Artificial C.S.F.

Batches of sterile artificial c.s.f. were prepared in 100 ml or 200 ml volumes by the Pharmacy Department at the Edinburgh Royal Infirmary.

The composition of the fluid perfused is shown in Table 2:1. In fact the substances marked with an asterisk, potassium chloride (sterile aqueous solution of 20% KCl) and sodium bicarbonate (solid) were not added until the morning of the perfusion.

The omission of potassium chloride until this late stage meant that experiments did not have to be restricted to a fixed time in relation to the delivery of the short-lived isotope: a relatively constant amount of ^{42}K could be added to the perfusion fluid (10 μc/100 ml) regardless of the specific activity of the

TABLE 2:1 : Composition of Artificial C.S.F.

Ionic Composition	meq/l
Sodium	151.18
Potassium	2.98
Calcium	2.85
Magnesium	2.00
Phosphate	0.58
Bicarbonate	25.6
Chloride	132.83

Constituents	g/l
Sodium Chloride	7.320
*Potassium Chloride	0.222
Magnesium Chloride	0.095
Calcium Chloride	0.158
Sodium Dihydrogen Phosphate	0.070
*Sodium Bicarbonate	2.150
Glucose	0.800

* see text (page116).

radioactive potassium chloride solution. Sodium bicarbonate was not added until after sterilisation in view of the difficulties encountered during heat sterilisation.

Solid inulin was added to the artificial c.s.f. to give an inflow concentration of the order of 150-250 µg/ml. Within this range the actual amount added was not critical since absolute measurements of inulin concentration were not required. Only the relative concentrations of the inflow and outflow fluids were necessary for analysis of the inulin data.

Preparation for ventricular-cisternal perfusion

Food was withheld from the animal for 18 hours prior to the experiment but water remained freely available up till the time of the experiment.

Approximately one hour before the start of an experiment the perfusion circuit was sterilised by pumping boiling water through it for 40 minutes and then allowing it to empty. The needles and stilettes appropriate to the dog being studied (see page112), were sterilised in boiling water, along with the three hollow steel needles which were to enter the bottle of sterile perfusion fluid.

Before commencing a perfusion the bottle of artificial c.s.f., containing ^{42}K and inulin in addition to the standard ingredients (Table 2:1), was equilibrated with 5% CO_2 in oxygen and this was continued throughout the perfusion. After 15 minutes to allow for complete equilibration the inflow circuit of the perfusion system

was primed with artificial c.s.f. Narrow range indicator paper was used to confirm that the pH of the inflow fluid was approximately 7.4.

The next step in the experimental procedure was to cannulate the ventricle and cisterna magna of the dog.

Ventricular Puncture

The skin overlying the ventricular guide tube on the side to be punctured was infiltrated with a small volume of lignocaine. (2%) The dog's head was steadied by an assistant and a needle of appropriate length, with its stilette in position, was pushed firmly through the skin into the guide tube and advanced to its full depth, after which the stilette was removed. A satisfactory placement within the ventricle was verified either by spontaneous welling up of c.s.f. into the cup of the needle, or by the appearance of c.s.f. when slight negative pressure was applied with a 1 ml syringe. When a free flow of c.s.f. had been established the luer-lock on the inflow tubing was firmly screwed onto the needle. The dog showed no more distress during this procedure than it would during a venepuncture.

Cisternal Puncture

As at the ventricular site the area of skin overlying the entrance to the guide tube was infiltrated with a small volume of lignocaine. Then, while an assistant supported the dog's head in a flexed position, a cisternal needle of appropriate length, with

its stilette in position, was pushed firmly through the skin into the guide tube and advanced for approximately half its length, after which the needle was given a slight bend. This bend in the needle acted as a spring to keep the needle firmly in position in the guide tube. After putting this bend in the needle a rotatory movement was used to advance the needle down the tube and through the atlantor occipital membrane into the cisterna magna. Satisfactory placement was verified when, on removal of the stilette, c.s.f. welled up into the cup of the cisternal needle. Occasionally it was necessary to apply slight negative pressure with a 1 ml syringe before c.s.f. could be drawn up into the cup of the needle. Once a free flow of c.s.f. had been established, the luer fitting on the outflow tubing was screwed firmly onto the hub of the needle.

The position of the needles relative to the ventricle and cisterna magna is shown in Fig. 1:6 .

Perfusion

Once both the ventricular and cisternal needles were in position and connected to the perfusion circuit (Fig. 2:3), perfusion tubing was secured to the back of the dog's neck with adhesive tape, in order to prevent tension in the tubing putting any direct pull on the needles. After a final check, a clock was started and the inflow and outflow pumps switched on. Having ensured that c.s.f. was flowing freely from the cisternal needle and that all was well on the inflow side the experimenter could then

FIG. 2:3 Photograph of dog A10(Dougal) during perfusion.

settle down to a routine of timing sample changes, keeping alert to any signs of blockage or leak in the system and amusing the dog, which in the case of an active dog was usually the most difficult task.

In control experiments the perfusion was continued for a period of 3-4 hours. In a drug experiment the perfusion proceeded as in a control experiment until a reasonable number of "steady-state" samples had been collected. The drug was then administered. Because of the considerable dead space in the outflow tubing (about 1.5 ml) the first one or two samples collected within ten minutes of giving the drug were not regarded as characteristic of the post-drug state.

If the drug being administered was an anaesthetic the dog had to be supported carefully during the induction period to make sure it did not collapse and disturb the perfusion needles. The unconscious dog was laid flat on its side and covered with a blanket. Since the perfusions were carried out in a warm room (25°C) no other measures were found to be necessary to maintain the dog's temperature at its normal level (37.8°C). As a check a rectal thermometer was routinely inserted after anaesthetising the dog.

From early experiments it was found that, at the flow rate of 300 $\pm$ 3 µl/min which was used in all these studies, a steady state was reached after 70-100 minutes of perfusion. During this steady state the concentrations of inulin and ^{42}K in the

outflow fluid were relatively constant.

At the end of the perfusion (between 3 and 4 hours usually) the pumps were switched off and the ventricular and cisternal needles gently removed. The dog was then returned to temperature-controlled kennels which were suitable for animals producing radioactive excreta. (In fact the excreta of these dogs did not reach detectable levels of radioactivity.)

Crystamycin (Glaxo) was given routinely by intramuscular injection, 1 ampoule (1,000,000 units benzyl penecillin and 0.5 g streptomycin) daily for three days.

The numbered samples of outflow fluid were removed from the fraction collector and placed along with three similar samples of inflow fluid ready for ^{42}K, inulin and total potassium assays. Because of the short half life of ^{42}K (12.5 hours) it was advisable to carry out the assay for this substance on the day of the perfusion. Once aliquots had been removed for the ^{42}K assay the samples were deep frozen until next day, at which time inulin and total potassium concentrations were estimated.

ANALYTICAL METHODOLOGY

Chemical Reagents and Drugs

Inulin	- British Drug Houses
^{42}K potassium chloride solution	- Radiochemical Centre, Amersham
Indol-3-yl acetic acid	- Koch Light Co., Ltd.
Ligno-caine ("Xylocaine")	- Astra Chemicals
Sodium thiopentone ("Pentothal")	- Abbott Laboratories Ltd.
Sodium pentobarbitone ("Nembutal")	- Abbott Laboratories Ltd.
Diazepam ("Valium")	- Roche Products Ltd.
Diphenylhydantoin ("Epanutin")	- Parke-Davis
Paraldehyde	- Evans Medical.

All reagents not mentioned above were analytical grade except hydrochloric acid which was micro-analytical grade (British Drug Houses). Distilled deionised water was used throughout.

The ^{42}K potassium chloride solution had a specific activity of 675 µc/meq at 13.00 hours on the day of delivery and could be used for up to three days after delivery.

Estimation of Inulin in Perfusion Fluid

Inulin in the inflow and outflow fluid samples was measured by an adaptation of the method of Heyrovsky (1956) in which no protein precipitation step was necessary because of the low levels of protein in the samples.

The assay procedure depends on the hydrolysis of the inulin to fructose units and their subsequent formation of a purple condensation product with indol-3-ylacetic acid.

With inflow concentrations of between 150 and 400 µg/ml and "steady state" outflow levels between 80 and 95% of that figure, an aliquot of 0.1 ml of perfusion fluid contained an appropriate amount of inulin for an assay within the linear range of the standard curve.

Procedure

An 0.1 ml aliquot of perfusion fluid was made up to 0.5 ml with water in a 15 ml glass tube and to this was added 0.1 ml indol-3-ylacetic acid solution (0.5% w/v in ethanol), and 4.0 ml concentrated hydrochloric acid. The contents of the tube were thoroughly mixed and then incubated at 40°C for 80 minutes. After cooling the tubes, the optical density of the purple solution was measured in a spectrophotometer (Unicam SP 500) at 530 mµ, against a water blank which contained all the reagents and had been taken through the incubation.

Over the range of inulin concentrations of the samples the optical density of the solution was directly related to the inulin concentration. It was not strictly necessary to include standards in the assay but duplicate standards containing 0-40 µg inulin per tube were always included with perfusion samples (Fig.2:4).

In the time taken to read the optical density of all the samples from a perfusion (up to 1.5 hours) it was found that the intensity of colour in the samples increased without a corresponding increase in the blank. Presumably this was because the hydrolysis of the inulin to fructose units was not completed by the

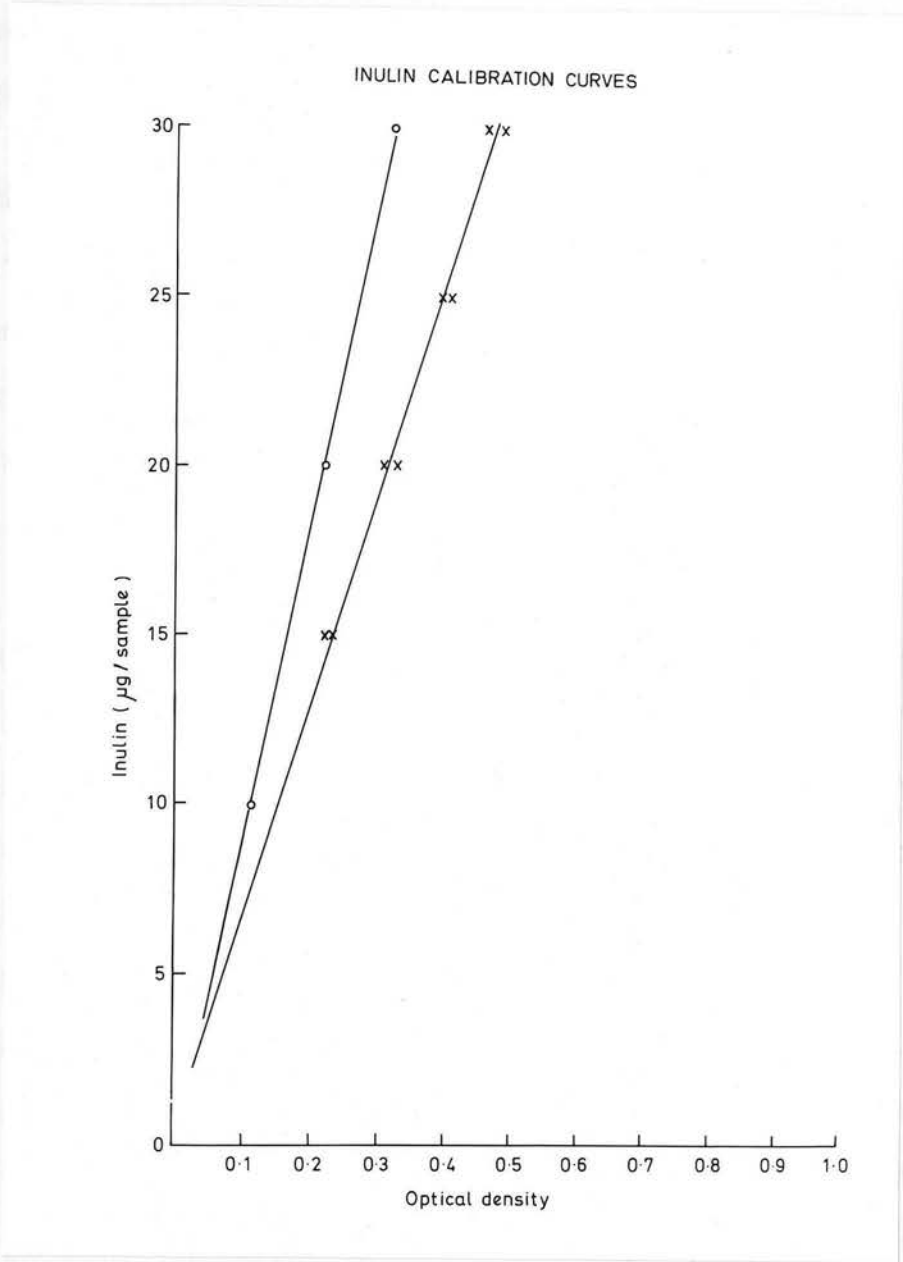


FIG. 2:4 Inulin Standard Curves

The curves demonstrate good linearity within the specified range of inulin concentrations.

incubation. A correction based on the average change in the standards over the time of optical density measurements was applied to all samples.

Estimation of ^{42}K in Perfusion Fluid

In aqueous solutions the high energy β emissions of ^{42}K generate Cerenkov radiation and this can be detected in a liquid scintillation counter. The efficiency of counting was determined by a channels ratio method (Moir, 1971) based on the original channels ratio method of Baillie (1960).

In the early experiments counting was done in the department of Medical Physics, Edinburgh Royal Infirmary on a Packard Series 3003 Tri-Cab Liquid Scintillation counter. Most of the counting was however done in Nuclear Chicago Mark II Liquid Scintillation Counter and the methodology to be described applies only to ^{42}K estimation using this instrument since the earlier methodology has been fully described by Moir (1971).

Because of the rapid decay of ^{42}K (half life 12.5 hours) the general level of activity in the samples tended to vary from experiment to experiment and the proportion of sample which was actually counted could be varied according to the estimated activity. Within each assay however the volume of sample counted remained constant. An aliquot of perfusion fluid (always between 0.2 and 1.0 ml) was made up to 10 ml, with water, in a 20 ml glass scintillation vial and counted at the settings shown in Table 2:2. The counts were first corrected for background

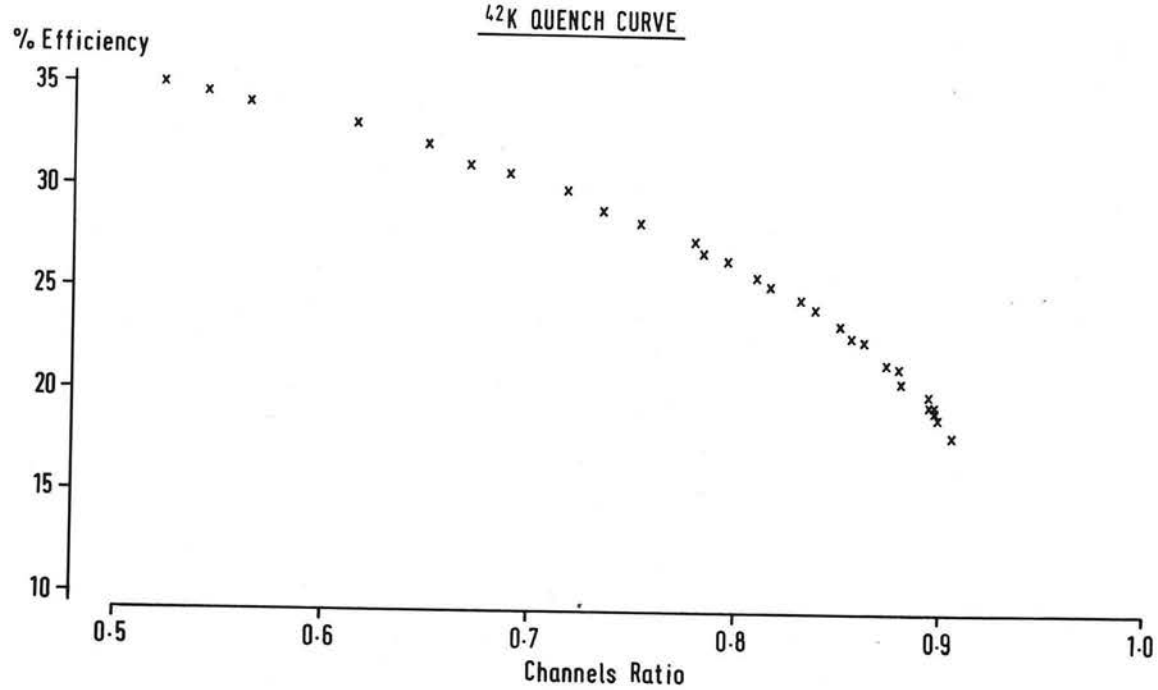


FIG. 2:5 Channels ratio quench* curve for Cerenkov counting of ^{42}K activity in aqueous solutions.

*Quenching agent - blood

and then for quenching, using the efficiency/channels ratio curve shown in Fig. 2:5.

TABLE 2:2 : Instrument[1] Settings for Cerenkov Counting of ^{42}K in Perfusion Fluid.

	Gain Setting	Discriminator Settings Lower	Upper
Channel 1[2]	A - 1000	05 -	30
Channel 2	A - 1000	05 -	99

[1] - Nuclear Chicago Mark II Liquid Scintillation Counter

[2] - Channels Ratio - Channel 1 / Channel 2.

On some occasions the radioactivity in the samples was low and the time taken to reach a satisfactory number of counts was long so that a correction factor had to be applied to the calculated counts in order to bring them to a common time of counting. For instance, if the samples were counted for 2 minutes each, the 30th sample would contain only 94.6% of the activity when the first sample was counted. The correction factors were obtained from isotope decay tables (Documenta Geigy, 1971).

Estimation of Total Potassium in Perfusion Fluid

Potassium was estimated by flame emission, with an SP 90 Atomic Absorptionmeter (Pye-Unicam) set in the emission mode.

An absorption filter was placed between the flame and the monochromator to reduce sodium interference to a minimum.

With the air/acetylene emission burner head in position the optimum conditions of assay were usually as follows:

Slit Width	:	0.08 mm
Wavelength	:	7665 mμ
Air Flow	:	4 l/min
Acetylene Flow	:	650 ml/min
Filter	:	Wratten No. 35 (Kodak)
Gain	:	High gain, position 3.
Burner Height	:	1.4 cm.

An 0.1 ml aliquot of sample was pipetted into a small polyethylene container (capacity 4 ml) and diluted to 2.1 ml with distilled deionised water. The emission of the solution was measured at the potassium resonance wavelength of 7665 mμ. Samples were read along with identical dilutions of potassium standards covering the range 2-4 meq/l. To account for any residual interference from the sodium in the samples the potassium standards were made up in a solution of sodium chloride (160 meq/l) with approximately the same sodium content as perfusion fluid and c.s.f. All standard solutions were stored in polyethylene bottles.

Over the range of potassium studied the emission was linearly related to the potassium concentration in the solution being atomised.

Estimation of pCO_2 of alveolar air

End alveolar air was obtained by a method based on that described by Travis, Wiley, Nechay and Maren (1964). One of the fingertips was cut out of a standard surgical glove and a hollow cardboard tube (diameter 1.5 cm, length 8 cm) was inserted into th finger, and the other four fingers were tied off. A small flexible butyl rubber tube (non-porous to CO_2), of internal diameter 0.25 cm, was then stuck to the inside of the tube, with one end facing into the glove and theother end free. This specially prepared glove was then fitted over the dog's nose and mouth and adjusted so that the cardboard tube was in direct apposition to the dog's nostrils, thus minimising the dead space. As the dog breathed out the expired gases in the rubber tube were sampled by a CO_2 gas analyser (Beckman LBI, Medical Gas Analyser). The content of CO_2 in the expired gases could be read off directly from this instrument. At the end of each breath the CO_2 content of the expired gases was stable and this value was taken as the best estimate for the CO_2 content of end alveolar air.

Estimation of pCO_2 and pH of C.S.F.

For the sampling of c.s.f. a small three way T-junction was inserted into the perfusion circuit immediately next to the outflow from the needle in the dog's cisterna magna. A small piece of butyl rubber tubing (non-porous to CO_2) was then connected up to the free arm of the junction which was normally kept closed by a small bulldog clip. Small glass capillaries (200 µl) could

then be inserted into the sampling arm and when the bulldog clip was released and placed on the perfusion outflow tubing beyond the junction, perfusate from the cisterna magna passed into the capillary.

From the capillary the sample was rapidly transferred to the sampling chamber of pH/pCO_2 analyser (Radiometer - Blood Micro System). After allowing the sample time to equilibrate to the operating temperature of the system (37 $\pm$ 0.03°C), the pH and CO_2 measurements were performed and the results were read off directly from the instrument.

THEORY

The basic equations necessary for kinetic analysis of open cerebroventricular systems were derived by Pappenheimer and his co-workers during their perfusion studies in the conscious goat (Pappenheimer, Heisey and Jordan, 1961; Heisey, Held and Pappenheimer 1962). Using fundamentally similar methods of analysis Cserr (1965), Bradbury and Davson (1965) and Katzman, Graziani, Kaplan and Escriva (1965) have investigated the transport of potassium between blood, brain and c.s.f.

In our own studies the perfusion data have been analysed in terms of a simple two compartmental model (Fig. 2:6), with the c.s.f. as one compartment and the brain and the blood as a composite second compartment.

Fig. 2:7 illustrates the time course of a typical perfusion experiment. In the first 70-100 minutes of a perfusion the endogenous c.s.f. in the dog's ventricular system is gradually displaced by perfusion fluid containing inulin and ^{42}K. This phase is represented by the increasing inulin and ^{42}K concentrations in the outflow fluid. When the process is complete a "steady state" is achieved in which the concentrations of ^{42}K and inulin in the outflow fluid remain constant, signifying that the rates of loss of ^{42}K and inulin from the system are also constant. The two compartmental analysis to be presented below applies only to data collected during this "steady-state" period.

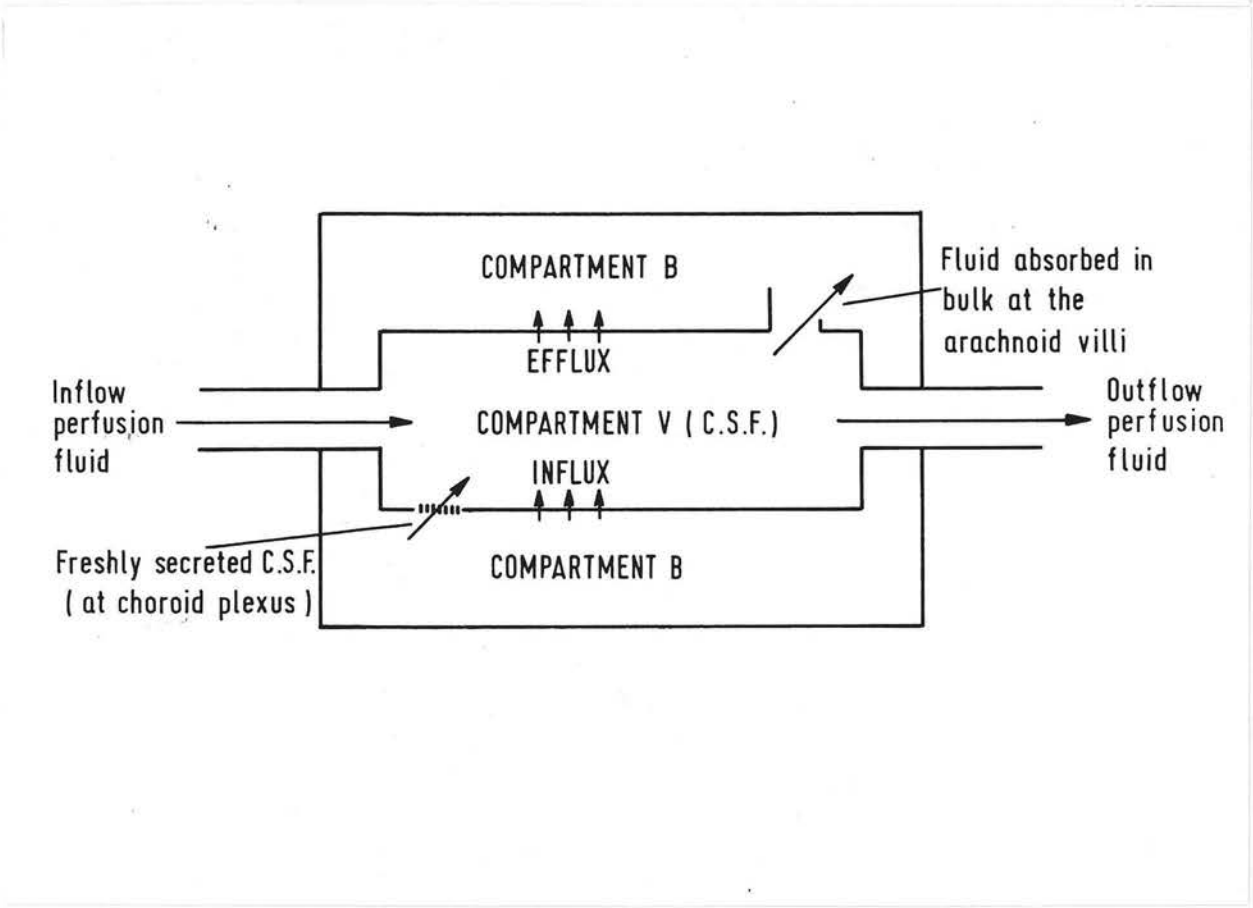


FIG. 2:6 Two-compartmental model of ventricular perfusion system. Compartment V is that part of the ventricular system which is being perfused. B is a composite compartment representing both blood and brain.

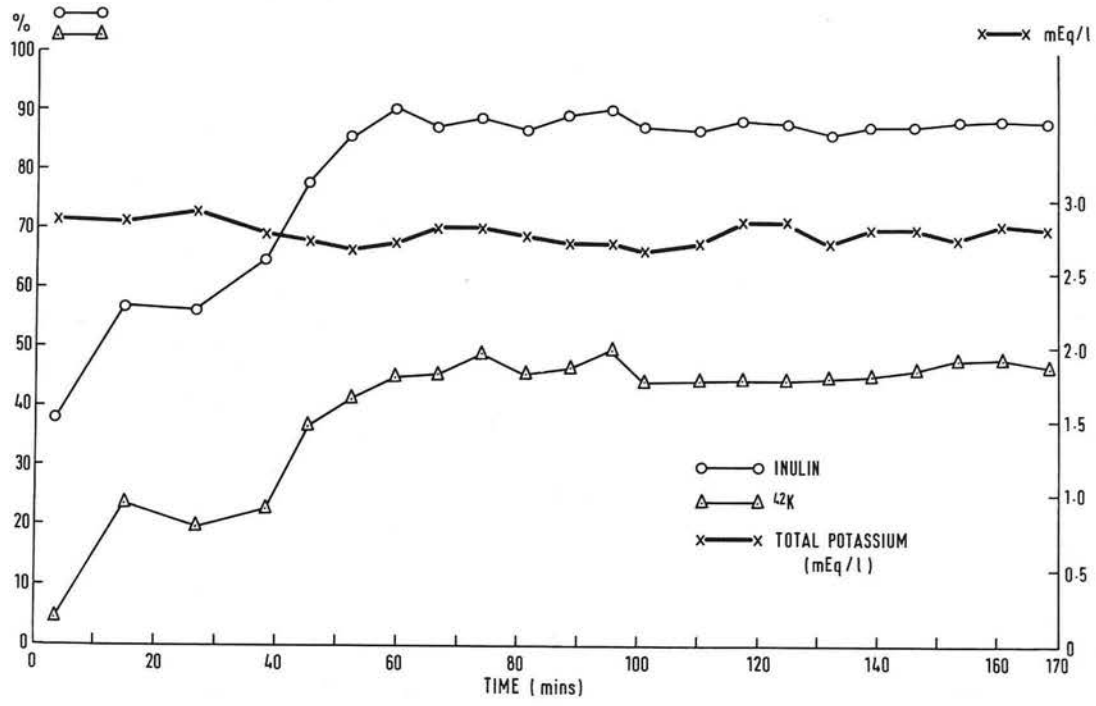


FIG. 2:7 Time course of a control perfusion; concentrations of inulin, ^{42}K and total potassium in the outflow perfusion fluid. The inulin and ^{42}K concentrations have been expressed as a percentage of their concentration in inflow fluid.

In this experiment the system reached a "steady-state" after a period of 60 to 80 minutes of perfusion (flow rate 0.300 ml/min).

Definitions

The flux of substance from one compartment to another is defined as the amount of substance which crosses the boundary in unit time. In the present experiments the area of the boundaries to the c.s.f. compartment perfused is not known but is assumed to remain constant throughout an experiment.

The loss of fluid from the system by bulk absorption refers to fluid which passes out of the cisterna magna into the subarachnoid spaces from where it drains back into the blood stream via the arachnoid villi. The clearance of substance x from compartment A by the pathway leading to compartment B is defined as the volume of A from which x is effectively removed per unit time via that pathway. It therefore has the dimensions of flow (volume of fluid/unit time), and when used in the term inulin clearance from the c.s.f. it actually refers to the flow of fluid out of the perfusion system into the subarachnoid spaces.

Symbols

$\dot{V}$	=	flow rate	- ml/min
C	=	concentration	- meq/ml
C^*	=	radioactivity	- d.p.m./ml
$\overline{C}$	=	mean concentration	- meq/ml
$\dot{C}lin$	=	inulin clearance from ventricular system	- ml/min
J	=	flux	- meq/min
J^*	=	flux (as applied to radioactive substance)	- d.p.m./min
N	=	net flux	- meq/min or d.p.m./min

Subscripts have been applied as follows:-

i - perfusion inflow fluid

o - perfusion outflow fluid

f - freshly secreted c.s.f.

a - fluid lost from system by bulk flow into the subarachnoid spaces

v - ventricular compartment

b - composite brain and blood compartment

These last two subscripts v and b when used in combination imply direction from the first to the second.

Rate of Formation of C.S.F. in the Ventricles - $\dot{V}f$

Assumption I - Diffusional losses of inulin from the c.s.f. compartment are negligible.

Then where C refers to inulin

$$\dot{V}i\ Ci = (\dot{V}i + \dot{V}f)\ Co$$

$$\therefore \quad \dot{V}f = \frac{\dot{V}i\ (Ci - Co)}{Co} \qquad \text{Eqn. (1)}$$

Rate of Bulk Absorption of Fluid - $\dot{V}a$

Assumption II - The volume and pressure within the c.s.f. compartment are constant.

Then $\dot{V}i + \dot{V}f = \dot{V}o + \dot{V}a$ Eqn. (2)

In our experiments $\dot{V}i = \dot{V}o$

$$\therefore \quad \dot{V}f = \dot{V}a$$

However the bulk absorption of c.s.f. may also be derived directly as below -

Assumption III - The fluid absorbed in bulk from the c.s.f. compartment has a similar inulin concentration to perfusion outflow fluid.

Assumption IV - The concentration of inulin in freshly secreted c.s.f. is negligible.

Then where C refers to inulin:

$$\dot{V}i\ Ci = \dot{V}o\ Co + \dot{V}a\ Co$$

$$\therefore \dot{V}a = \frac{\dot{V}i\ Ci - \dot{V}o\ Co}{Co} \qquad \text{Eqn. (3)}$$

As $\dot{V}i = \dot{V}o$ in our system eqn. (3) is identical with eqn. (1). $\dot{V}a$ (bulk absorption) has been termed $\dot{C}l$ in (Inulin Clearance) by Pappenheimer and colleagues, where clearance is expressed in ml of outflowing perfusate.

Potassium Data

Once the rate of formation/bulk absorption of c.s.f. have been calculated from the inulin data it is possible to split up the steady-state loss of ^{42}K from the system into that fraction which is lost by bulk absorption of fluid, and that fraction which passes either into the brain across the ependymal barrier or into the blood across the choroid plexus epithelium.

The net flux of substance across the cellular barriers making up the boundaries of the c.s.f. compartment is equal to the flux of substance out of c.s.f. (i.e. efflux) minus the flux of substance into the c.s.f. (i.e. influx).

i.e. $N = Jvb - Jbv$ Eqn. (4)

and $\dot{V}i\ Ci + \dot{V}f\ Cf + Jbv = \dot{V}o\ Co + \dot{V}a\ Co + Jvb$ Eqn. (5)

$\therefore N = \dot{V}i\ Ci - \dot{V}o\ Co + \dot{V}f\ Cf - \dot{V}a\ Co$ Eqn. (6)

In the case where ^{42}K is present in tracer amounts in the perfusion fluid -

Assumption V - During the time course of the experiment there is no appreciable labelling of brain or plasma, therefore:

$$\overset{*}{Jbv} = 0$$

and $$\overset{*}{Cf} = 0$$

$\therefore$ Combining Eqn. (4) with Eqn. (6)

$$\overset{*}{Jvb} = \dot{V}i\ \overset{*}{Ci} - \dot{V}o\ \overset{*}{Co} - \dot{V}a\ \overset{*}{Co}$$

$$= \dot{V}i\ \overset{*}{Ci} - (\dot{V}o + \dot{V}a)\ \overset{*}{Co}$$ Eqn. (7)

Assumption VI - The tracer atoms of ^{42}K behave in manner representative of the parent species.

To convert this efflux in terms of tracer ^{42}K to an efflux in terms of potassium the terms on the right of Eqn. (7) were divided by the mean specific activity of the ventricular compartment.

Assumption VII - The mean specific activity of the c.s.f. compartment can be represented by the arithmetic mean of the specific activities of the inflow and outflow fluid.

∴ For potassium

$$Jvb = \frac{\dot{V}i\,\overset{*}{C}i - (\dot{V}o + \dot{V}a)\,\overset{*}{C}o}{\left(\frac{\overset{*}{C}i}{Ci} + \frac{\overset{*}{C}o}{Co}\right)} \times 0.5 \qquad \text{Eqn. (8)}$$

Returning now to Eqn. (5)

$$\dot{V}i\ Ci + \dot{V}f\ Cf + Jbv = \dot{V}o\ Co + \dot{V}a\ Co + Jvb \qquad \text{Eqn. (5)}$$

we can see that all the terms are known for potassium except Cf and Jbv.

Assumption VIII - The concentration of potassium in newly secreted c.s.f. in the dog has a constant value of 3.0 meq/l (Ames, Sakanoue, and Endo, 1964; Cserr, 1965).

Then $Jbv = \dot{V}o\ Co - \dot{V}i\ Ci + \dot{V}a\ Co - \dot{V}f.3.0 + Jvb$ Eqn. (9)

Both the efflux (Jvb) and influx (Jbv) of potassium from and into the c.s.f. compartment have the units of ~~meq/l.~~ meq/min.

"Clearance" Co-efficients

In the studies of Cserr (1965), Bradbury and Davson (1965) and Katzman, Graziani, Kaplan and Escriva (1965) the efflux of potassium from the system was divided by the mean concentration

of potassium in the c.s.f. compartment to give an efflux co-efficient having the dimensions of clearance, namely ml/min. In order to compare our results with the results of these earlier studies we have also calculated these efflux co-efficient terms.

$$\text{Potassium Efflux Co-efficient} = \frac{Jvb}{Cv}$$

$$= \frac{\dot{V}i\ \overset{*}{C}i - (\dot{V}o + \dot{V}a)\ \overset{*}{C}o}{\overset{*}{\bar{C}}} \quad \text{Eqn. (10)}$$

where $\overset{*}{\bar{C}} = \overset{*}{C}o + 0.37\ (\overset{*}{C}i - \overset{*}{C}o)$ (logarithmic mean)

In these earlier studies this estimate of $\bar{C}$ for the c.s.f. compartment was justified on the basis of the ventricular system being similar to a hollow tube of uniform diameter. However, the concentrations of ^{42}K and inulin in the outflowing perfusate during the early phase of a perfusion are not well fitted by single exponentials so that the assumption made in calculating $\bar{C}$ as the logarithmic mean does not seem justified.

In analysing our own data we have used both this method of calculating the mean activity of c.s.f. with respect to ^{42}K, and the more straightforward method of taking a simple arithmetic mean of inflow and outflow fluids.

RESULTS

Behaviour during Perfusion

The dogs were contained within a box of opaque perspex, the side of which could be completely removed. This box which is shown in Fig. 2:1, was sufficiently large to allow the dogs to move about and lie down and the open side gave ready access to the dog for procedures such as intravenous injections.

During a perfusion the dog's behaviour was always normal (Fig. 2:3), provided there were no technical difficulties with the perfusion. One or two of the dogs would sit quietly throughout the whole procedure but more usually the dog would expect to be petted by the experimenter and would maintain great interest in all the activity in the laboratory. Towards the end of a long (3-4 hour) perfusion it was not uncommon to find the more active dogs showing signs of a slightly stiff neck, but this appeared to resolve itself spontaneously within a few hours of stopping the perfusion.

Pressure Changes in the Ventricular System during Perfusion

As was mentioned in the methods section (page 111) this open perfusion technique differed from previous open systems (e.g. Pappenheimer, Heisey, Jordan and Downer, 1962) in that fluid was pumped out of, as well as into the ventricular system. Theoretically, provided the rate of perfusion is not too rapid, the dog should be able to control the pressure within the ventricular

system by its normal physiological mechanisms. To check this point low pressure transducers (Sanborn) were connected to the inflow and outflow sides of the perfusion circuit, as near as possible to the ventricular and cisternal needles, and pressure changes within the system recorded. Fig. 2:8 illustrates the typical pressure changes which were observed. As can be seen, the pump, when working at a flow rate of 0.3 ml/min, decreased cisternal pressure by approximately 3 cm of water. This is the order of pressure change in the c.s.f. system which was produced by a spontaneous deep breath. The ventricular c.s.f. pressure was increased by a similar amount during operation of the perfusion pump.

During later experiments the Watson-Marlow pump was replaced with a Technicon Auto-Analyser proportioning pump and the pressures developed within the system with this pump were again checked and found to be of the same order. On this occasion the pressure changes were recorded on a differential pressure gauge (Sifam).

Presentation of Data

The initial computer analyses for each individual perfusion experiment are shown in Appendix I of this thesis.

During the early stages of this project, while the experimental procedure was being developed, a simple graphical presentation of the primary data was found most useful. The concentrations of ^{42}K, inulin and total potassium in each sample of outflow fluid were plotted against the time after starting perfusion. From such a graph (Fig. 2:7) it was possible to see at a glance how the ^{42}K and inulin concentrations in the outflow rose to

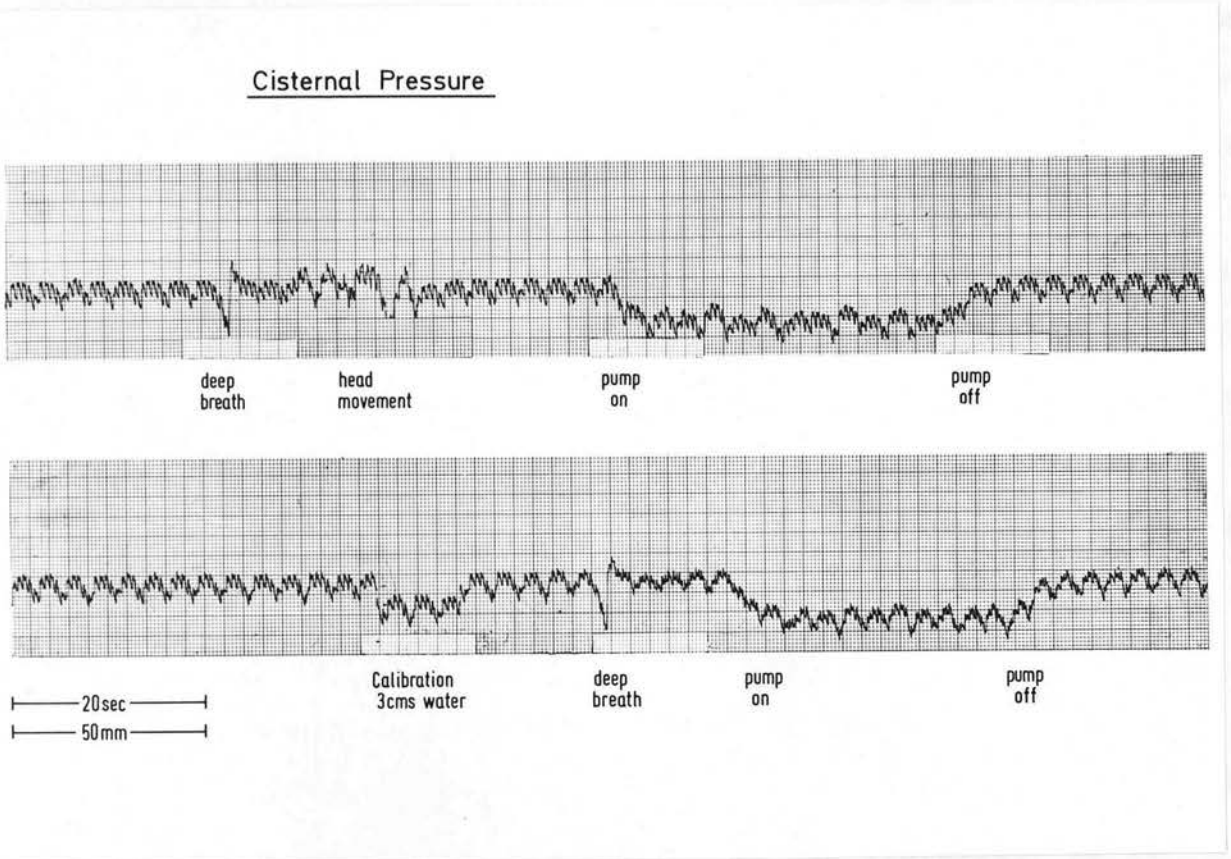


FIG. 2:8 : Effect of perfusion on pressure within a dog's c.s.f. system.

At the point on the trace marked "calibration 3 cms water" an offset voltage was applied to the transducer output which produced an apparent decrease in cisternal pressure to the extent of 30 mm water.

"steady state" levels over a period of 60-90 minutes.

For a precise analysis of "steady state" fluxes of potassium into and out of the c.s.f. compartment, the primary data were subjected to the mathematical treatment outlined in the previous theoretical section. The largely repetitive mathematical calculations were carried out by way of a specially designed computer programme (see Appendix I). The computed results from a typical control experiment are shown in Table 2:3.

Columns I and II specify the outflow sample number and the time after the start of the perfusion at which it was collected. The first few samples shown, with a collection time of zero, are in fact samples of inflow fluid and in their case the "K out" column refers to the potassium concentration of the inflow fluid.

Column III, marked inulin clearance, ml/min, shows the inulin clearance at the time of collection of the particular sample. As has been demonstrated in the theoretical section (Eqn. (3) in the theoretical section) this is equivalent to the rate of bulk absorption of fluid. In the conditions of our particular perfusion system, where the inflow perfusion rate is equal to the outflow perfusion rate (i.e. $\mathring{V}i = \mathring{V}o$), inulin clearance is also equivalent to the rate of formation of c.s.f. within the ventricular system (Eqn. 1 - theoretical section).

Column IV shows the concentration of total potassium in the outflow perfusion fluid (with the exception of the first few samples where it refers to inflow fluid as explained above)

DATE- 4/3/69 DOG-A2(ELMO) PROCEDURE- CONTROL

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN
26	0.0	0.0000	2.78	0.359	0.000	0.0000	0.0000	0.0000	0.0000
25	0.0	0.0000	2.78	0.364	0.000	0.0000	0.0000	0.0000	0.0000
24	0.0	0.0000	2.78	0.362	0.000	0.0000	0.0000	0.0000	0.0000
1	3.4	0.4895	2.85	0.363	0.041	1.4319	1.3795	0.5146	0.6767
2	15.0	0.2300	2.83	0.347	0.229	0.8100	0.7859	0.2904	0.3470
3	27.0	0.2386	2.90	0.370	0.188	0.9360	0.9481	0.3345	0.4067
4	38.4	0.1651	2.75	0.364	0.221	0.8972	0.8469	0.3234	0.3877
5	45.4	0.0871	2.70	0.364	0.358	0.6558	0.6057	0.2377	0.2711
6	52.5	0.0513	2.65	0.357	0.405	0.6154	0.5585	0.2245	0.2523
7	59.8	0.0333	2.70	0.352	0.439	0.5891	0.5551	0.2138	0.2380
8	67.0	0.0448	2.80	0.356	0.445	0.5647	0.5617	0.2027	0.2251
9	74.3	0.0390	2.80	0.362	0.480	0.5164	0.5146	0.1853	0.2039
10	81.6	0.0460	2.75	0.358	0.444	0.5615	0.5410	0.2027	0.2252
11	88.7	0.0382	2.70	0.353	0.455	0.5534	0.5179	0.2009	0.2226
12	95.9	0.0333	2.70	0.366	0.489	0.5071	0.4731	0.1842	0.2023
13	101.3	0.0448	2.65	0.347	0.434	0.5750	0.5203	0.2099	0.2339
14	110.4	0.0468	2.70	0.358	0.434	0.5739	0.5358	0.2083	0.2321
15	117.6	0.0409	2.84	0.352	0.439	0.5851	0.5955	0.2091	0.2327
16	124.8	0.0417	2.84	0.359	0.436	0.5887	0.6000	0.2104	0.2343
17	132.0	0.0488	2.70	0.366	0.439	0.5616	0.5230	0.2039	0.2268
18	139.2	0.0432	2.79	0.357	0.441	0.5742	0.5681	0.2063	0.2295
19	146.4	0.0432	2.80	0.361	0.451	0.5568	0.5541	0.1998	0.2216
20	153.6	0.0409	2.73	0.356	0.471	0.5243	0.4983	0.1897	0.2093
21	160.9	0.0394	2.83	0.355	0.474	0.5286	0.5369	0.1891	0.2084
22	168.1	0.0417	2.79	0.352	0.457	0.5494	0.5436	0.1974	0.2186

while column V, "K42 channels ratio", has been included simply as a check on the radioactivity measurements.

Column VI, "K42 out/in ratio", is the ratio of ^{42}K activity in outflow fluid to the activity in inflow fluid.

Column VII and VIII refer to the efflux (Jvb) and influx (Jbv) of potassium into and out of the c.s.f. compartment. These parameters (Eqns. 8 and 9 in the theoretical section) have been corrected for loss of ^{42}K by bulk flow into the sub-arachnoid spaces, and refer to fluxes across the ependymal barrier of the brain and the choroid epithelial barrier of the blood. In the present study we have not differentiated between these two possibilities.

The last column, "K(2) efflux ml/min", is the potassium efflux coefficient of Cserr (1965), Bradbury and Davson (1965) and Katzman et al. (1965) (Eqn. 10 in the theoretical section). The preceeding column, "K(1) efflux ml/min", is the same parameter except that in this case the mean activity of the c.s.f. compartment has been calculated as the arithmetic mean of the inflow and outflow fluids rather than as the logarithmic mean which was employed in the calculation of the K(2) coefficient.

As one might expect from examination of the primary data (Fig. 2:7) the computed parameters, inulin clearance and potassium efflux and influx, reach steady state levels about 70 minutes after the start of the perfusion (Table 2:3).

Analysis of Drug Effects

It was originally intended that values for efflux and influx of potassium collected in the "steady state" period before the administration of the drug should act as controls for the post-drug "steady-state" values. However from examination of the control data it became apparent that flux values during the "steady-state" did not in fact remain constant but tended to decrease throughout the time of a perfusion. This may be because the assumption of negligible labelling of the brain compartment becomes increasingly less valid as perfusion time increases. Whatever the reason for this trend towards lower efflux and influx values with increasing time of perfusion, it was assumed to occur in drug experiments independently of any specific drug effect.

To assess the extent of the deviations from "steady-state" the potassium flux values from five control conscious perfusion experiments were plotted against perfusion time, over the approximate "steady-state" period. To standardise the data from the five experiments the flux values for each individual sample within an experiment were expressed as a percentage of the mean control value in that experiment, during the period 90-120 minutes. The data from the five different experiments could then be plotted on the one scale and the composite graphs for control efflux and influx data are shown in Fig. 2:9 and Fig. 2:10 respectively.

Inspection of Fig. 2:9 suggested that the efflux data could be adequately represented by a simple quadratic expression.

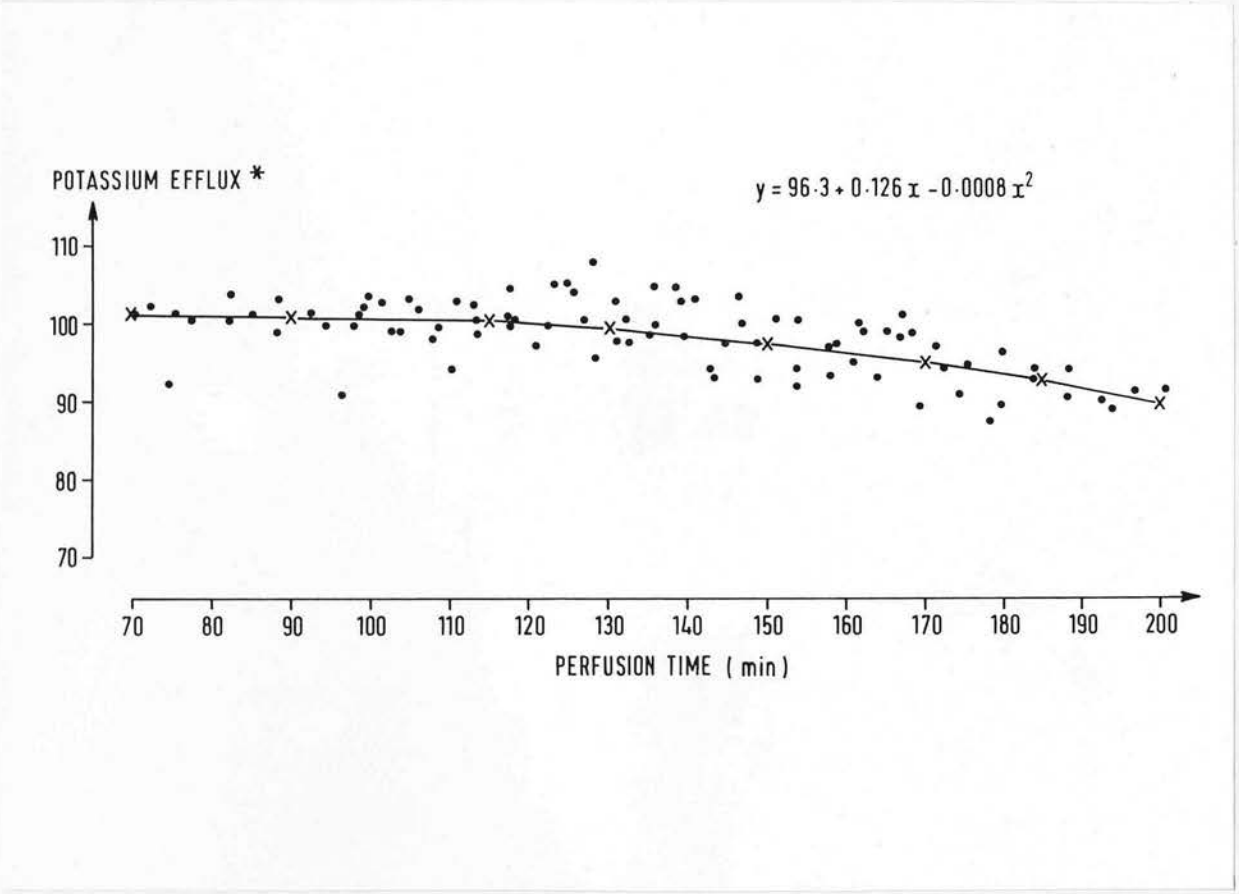


FIG. 2:9 Composite graph showing c.s.f. potassium efflux data from five control experiments.

The efflux data for each experiment has been expressed as a percentage of the mean efflux in that experiment during the time period 90-120 minutes (see text p.148).

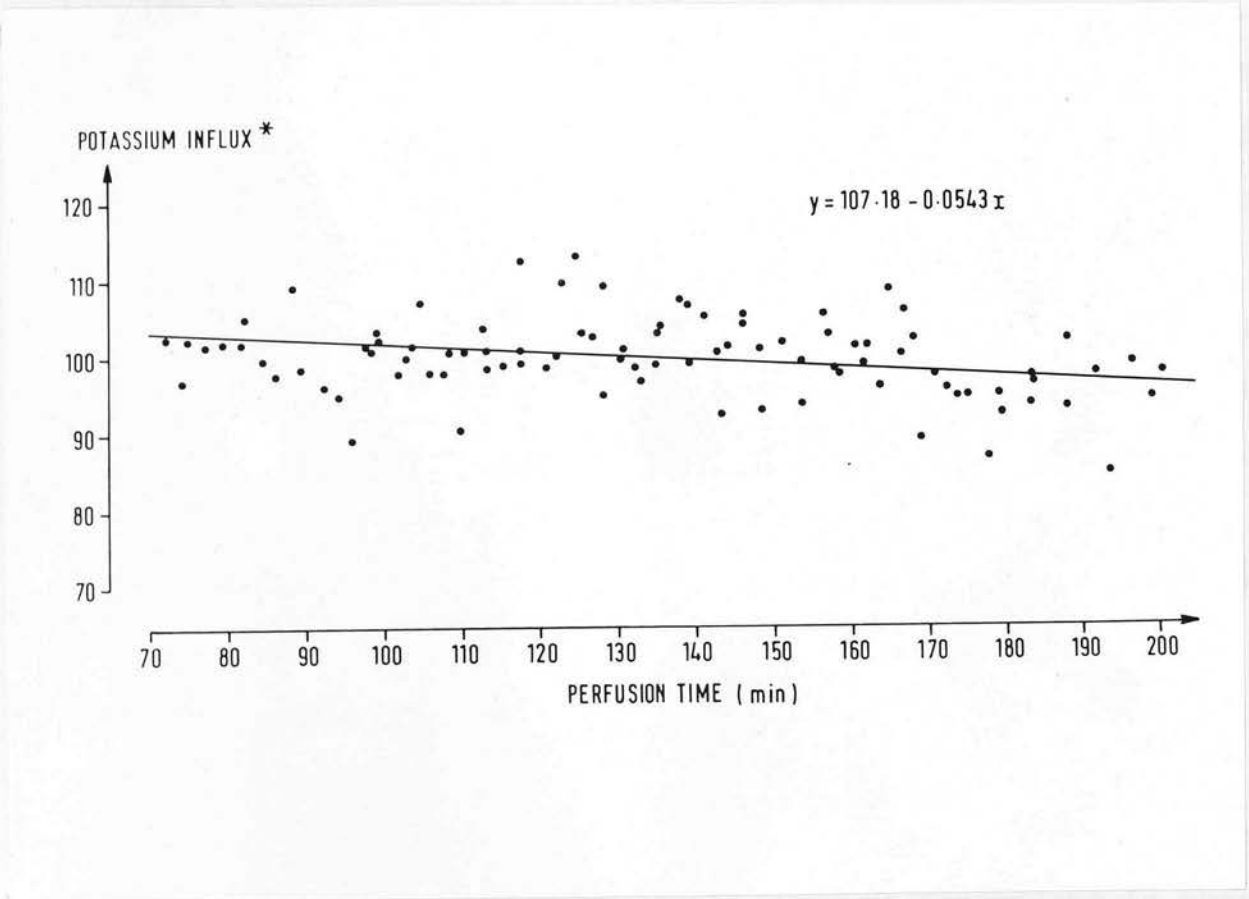


FIG. 2:10 Composite graph showing c.s.f. potassium influx data from five control experiments.

The influx data for each experiment has been expressed as a percentage of the mean influx in that experiment during the time period 90-120 minutes (see text p.148).

The fitted curve is shown superimposed on the individual efflux values. When an attempt was made to express the influx data (Fig. 2:10), as a simple quadratic the square term of the resultant expression was so small as to be negligible. The influx data was therefore represented as a simple straight line.

These expressions, calculated from the pooled results of five control perfusions, could then be fitted to data from the control periods of drug experiments. Then by extrapolation it was possible to calculate hypothetical flux values which might have been obtained if no drug had been given. If these hypothetical values were calculated at the times for which there were actual flux estimations it was possible to compare post-drug values with hypothetical non-drug values. Since such a comparison was made whenever post-drug flux values were known it was possible to obtain an estimate of the size of the drug effect and of the error variation involved. The real and the hypothetical flux values were subjected to a paired t-test analysis. The procedure has been illustrated below by considering an experiment in which pentobarbitone was administered 136 minutes after the start of a conscious perfusion.

The computed print-out is shown in Table 2:4. To simplify the explanation only efflux data will be analysed. The "steady-state" period has been considered to start with sample 18 (94.7 min), by which time the inulin clearance and potassium efflux have stabilised. As in most experiments the transitional

DATE- 20/4/71 DOG- A7(JAFFA) PROCEDURE- PENTOBARBITONE — 136 min

EXPT. 6

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
0	0.0	0.0000	2.72	0.641	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.72	0.641	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.72	0.638	0.000	0.0000	0.0000	0.0000	0.0000	
1	9.5	2.2641	3.08	0.390	0.001	1.6206	1.9097	0.5958	0.8046	
3	19.0	0.3897	3.03	0.650	0.139	0.9866	1.0913	0.3582	0.4458	
6	35.0	0.1950	3.07	0.649	0.193	0.9503	1.0689	0.3429	0.4161	
7	40.0	0.1348	3.02	0.642	0.232	0.8956	0.9883	0.3231	0.3856	
8	45.0	0.1149	3.09	0.648	0.247	0.8837	1.0051	0.3172	0.3763	
9	49.0	0.1000	3.11	0.646	0.251	0.8910	1.0190	0.3193	0.3782	
10	54.1	0.0822	2.98	0.642	0.267	0.8663	0.9427	0.3127	0.3680	
11	59.2	0.0717	3.00	0.641	0.258	0.8998	0.9838	0.3245	0.3833	
12	64.3	0.0632	3.02	0.647	0.268	0.8883	0.9796	0.3197	0.3762	
13	69.3	0.0681	3.02	0.642	0.263	0.8937	0.9850	0.3218	0.3793	
14	74.4	0.0538	3.04	0.647	0.278	0.8787	0.9768	0.3156	0.3700	
15	79.5	0.0555	3.03	0.642	0.267	0.9011	0.9957	0.3241	0.3816	
16	84.6	0.0563	3.03	0.645	0.260	0.9140	1.0086	0.3289	0.3881	
17	89.7	0.0505	3.06	0.650	0.279	0.8811	0.9862	0.3161	0.3704	
18	94.7	0.0448	2.96	0.652	0.305	0.8287	0.8990	0.2989	0.3470	
19	99.7	0.0448	3.06	0.647	0.289	0.8670	0.9717	0.3108	0.3628	
20	104.7	0.0429	3.03	0.653	0.263	0.9227	1.0170	0.3320	0.3913	
21	109.7	0.0440	3.07	0.645	0.262	0.9275	1.0356	0.3329	0.3927	
22	114.7	0.0440	3.09	0.646	0.296	0.8556	0.9706	0.3060	0.3563	Control
23	119.9	0.0440	3.08	0.641	0.267	0.9164	1.0280	0.3286	0.3868	
24	125.0	0.0382	3.08	0.648	0.323	0.8079	0.9190	0.2886	0.3329	
25	130.1	0.0311	3.05	0.647	0.339	0.7835	0.8841	0.2802	0.3214	
26	135.3	0.0275	3.06	0.651	0.317	0.8329	0.9365	0.2980	0.3445	
27	140.7	0.0243	3.05	0.647	0.310	0.8498	0.9500	0.3044	0.3527	
28	145.7	0.0356	2.97	0.644	0.297	0.8572	0.9312	0.3091	0.3598	
29	151.1	0.0390	2.99	0.650	0.309	0.8300	0.9106	0.2987	0.3462	
30	157.0	0.0371	2.94	0.644	0.322	0.8027	0.8865	0.2897	0.3343	
31	162.2	0.0344	2.96	0.637	0.352	0.7498	0.8204	0.2698	0.3083	
32	167.3	0.0352	3.01	0.642	0.371	0.7150	0.8023	0.2560	0.2906	
33	172.4	0.0330	3.00	0.647	0.370	0.7209	0.8049	0.2584	0.2935	
34	177.5	0.0319	3.00	0.640	0.378	0.7072	0.7912	0.2533	0.2870	
35	182.7	0.0337	3.04	0.646	0.382	0.7003	0.7977	0.2500	0.2829	Treatment
36	187.8	0.0289	2.92	0.637	0.381	0.7022	0.7599	0.2533	0.2867	
37	193.0	0.0275	2.97	0.636	0.378	0.7113	0.7854	0.2555	0.2894	
38	198.2	0.0326	2.99	0.643	0.381	0.7001	0.7808	0.2510	0.2841	
39	203.4	0.0315	3.00	0.648	0.387	0.6920	0.7760	0.2478	0.2800	
40	208.7	0.0226	3.00	0.647	0.398	0.6858	0.7698	0.2454	0.2763	
41	214.8	0.0229	3.00	0.644	0.397	0.6869	0.7709	0.2458	0.2769	
42	220.0	0.0330	3.00	0.637	0.388	0.6872	0.7712	0.2461	0.2779	

sample collected just after administering the drug, in this case sample 27, has been omitted from the analysis. The mean potassium efflux in the post-drug period was only 84.9 $\pm$ 6.4% of the mean efflux in the pre-drug control level. The following analysis was carried out to investigate whether this was a specific drug effect.

Mean time of collection of pre-drug control samples

= 114.9 min

From standard control curve

Potassium efflux at 114.9 min = 96.3 + 114.9 (0.126)

$- (114.9)^2 \times 0.00079$ units = 100.35 units.

In fact mean control potassium efflux = 0.8602 meq/min.

$\therefore$ 100 units on standard control curve $\equiv$ 0.8572 meq/min

Let us now consider sample 35 collected at 182.7 min.

From the standard curve potassium efflux at this time should be

$= 96.3 + 0.126\ (182.7) - 0.00079\ (182.7)^2$ units

= 92.95 units

which in this particular experiment would be

$\equiv 92.95 \times 0.8572$ meq/~~ml~~ min

= 0.7968 meq/~~ml~~ min

Actual observed value = 0.7003 meq/~~ml~~ min

Difference = -0.0965 meq/~~ml~~ min

If the recorded values for all the samples are compared in this way with hypothetical control values we can prepare a table of differences (Table 2:5).

The differences were then subjected to a paired t-test analysis.

Mean difference	=	-0.0636
Variance	=	0.00114
Degrees of freedom	=	14

$$t = -7.02$$

This t value is significant at level of probability < 0.001 and it indicates that in this experiment pentobarbitone has significantly lowered the efflux of potassium from the c.s.f.

The analysis of influx data follows exactly the same pattern.

TABLE 2:5 : Analysis* of Drug Effect on Potassium Efflux

Time of Sample (min)	Hypothetical Control Flux (meq/min)	Actual Flux (meq/min)	Difference (meq/min)
145.7	0.8391	0.8572	+0.0181
151.1	0.8341	0.8300	-0.0041
157.0	0.8282	0.8027	-0.0255
162.2	0.8226	0.7498	-0.0728
167.3	0.8167	0.7150	-0.1017
172.4	0.8105	0.7209	-0.0896
177.5	0.8039	0.7072	-0.0967
182.7	0.7968	0.7003	-0.0965
187.8	0.7895	0.7022	-0.0873
193.0	0.7817	0.7113	-0.0704
198.2	0.7736	0.7001	-0.0735
203.4	0.7650	0.6920	-0.0730
208.7	0.7600	0.6858	-0.0702
214.8	0.7451	0.6869	-0.0582
220	0.7354	0.6872	-0.0482

* See text pages 148 to 154 .

Sodium Thiopentone : Effect on C.S.F. Potassium Fluxes

Sodium thiopentone (50 mg/ml solution) was administered intravenously in a dose which rapidly induced in the dog a state of light anaesthesia. In this state the corneal reflex was still present but the dog barely reacted to a moderately painful pressure stimulus applied between the toes. The required dose was found to be approximately 23 mg/kg. As the perfusion progressed it was necessary in some dogs to give supplementary intravenous doses of drug to maintain anaesthesia at a constant level.

Table 2:6 summarises the potassium efflux data from five experiments in which sodium thiopentone anaesthesia was induced. In experiment I the anaesthetic reduced the potassium efflux by 31.6%. As the individual sample data plotted in Fig. 2:11 illustrates, this effect was seen almost immediately. The only other experiment in which a significant change was seen was experiment 4 where there was an 8.7% ~~clearance~~ reduction in potassium efflux. In experiments 2, 3, and 5 there was no significant change in potassium efflux.

Table 2:7 summarises the potassium influx data from the same five experiments. As in the case of the efflux data, the most significant change was observed in experiment 1, where there was a 34% decrease in the influx of potassium into the c.s.f. In experiment 4 also, a significant decrease (15%) in influx was observed. In experiment 2 there was a barely significant

TABLE 2: 6 : Efflux of Potassium from the C.S.F. : Effect of Sodium Thiopentone Anaesthesia

Expt.	Date	Dog	Potassium Efflux Pre-Drug (meq/min)	Observed Post-Drug Potassium Efflux as % of Pre-Drug Value	Change in [2] Potassium Efflux due to drug (meq/min)	% Change in [3] Potassium Efflux due to drug	Effect of drug on efflux	t-value[4]	Significance of drug effect
1	1/4/69	A 2	0.6219 ± 0.0159(6)[1]	62.9 ± 6.0%(10)	-0.1964 ± 0.0265(10)	31.6%	↓	21.98	< 0.001
2	8/4/69	A 2	0.6354 ± 0.0144(6)	92.0 ± 4.6%(10)	-0.0022 ± 0.0195(10)	0.4%	↓	0.34	-
3	28/10/70	A10	0.7414 ± 0.0246(5)	92.6 ± 8.1%(8)	-0.0015 ± 0.0227(8)	0.2%	↓	0.17	-
4	5/11/70	A 1	0.6124 ± 0.0364(9)	83.9 ± 3.0%(6)	-0.0535 ± 0.0248(6)	8.7%	↓	4.81	< 0.01
5	25/11/70	A 1	0.5976 ± 0.0263(7)	94.8 ± 6.5%(8)	+0.0066 ± 0.0218(8)	1.1%	↑	0.80	-

[1] - mean ± standard deviation; no. of estimates in parentheses.

[2] - this column represents the mean difference between hypothetical control values extrapolated from the pre-drug period and the actual post-drug values observed (see full explanation in text, page 148).

[3] - in this column the mean difference in potassium efflux is presented as a percentage of the pre-drug efflux value.

[4] - Paired t-test between hypothetical control values and actual observed values (paired with respect to time of estimates).

TABLE 2: 7 : Influx of Potassium from the C.S.F. : Effect of Sodium Thiopentone Anaesthesia

Expt.	Date	Dog	Potassium Influx Pre-Drug (meq/min)	Observed Post-Drug Potassium Influx as % of Pre-Drug Value	Change in[2] Potassium Influx due to drug (meq/min)	% Change in[3] Potassium Influx due to drug	Effect of drug on influx	4 t-value	Significance of drug effect
1	1/4/69	A 2	0.5905 ± 0.0110[1](6)	61.7 ± 5.9%(10)	-0.2034 ± 0.0292(10)	34%	↓	20.93	< 0.001
2	8/4/69	A 2	0.5891 ± 0.0173(6)	92.4 ± 5.2%(10)	-0.0223 ± 0.0259(10)	3.8%	↓	2.59	< 0.05
3	25/10/70	A10	0.5887 ± 0.0311(5)	95.6 ± 5.1%(8)	-0.0079 ± 0.0249(8)	1.3%	↓	0.84	-
4	5/11/70	A 1	0.6577 ± 0.0414(9)	81.5 ± 4.3%(6)	-0.0985 ± 0.0276(6)	15%	↓	7.98	< 0.001
5	25/11/70	A 1	0.6945 ± 0.0394(7)	92.8 ± 6.4%(8)	-0.0258 ± 0.0355(8)	3.7%	↓	1.92	-

[1] mean ± standard deviation; no. of estimates in parentheses.

[2] this column represents the mean difference between hypothetical control values extrapolated from the pre-drug period and the actual post-drug values observed (see full explanation in text, page 148).

[3] in this column the mean difference in potassium influx is presented as a percentage of the pre-drug influx value.

[4] Paired t-test between hypothetical control values and actual observed values (paired with respect to time of estimates).

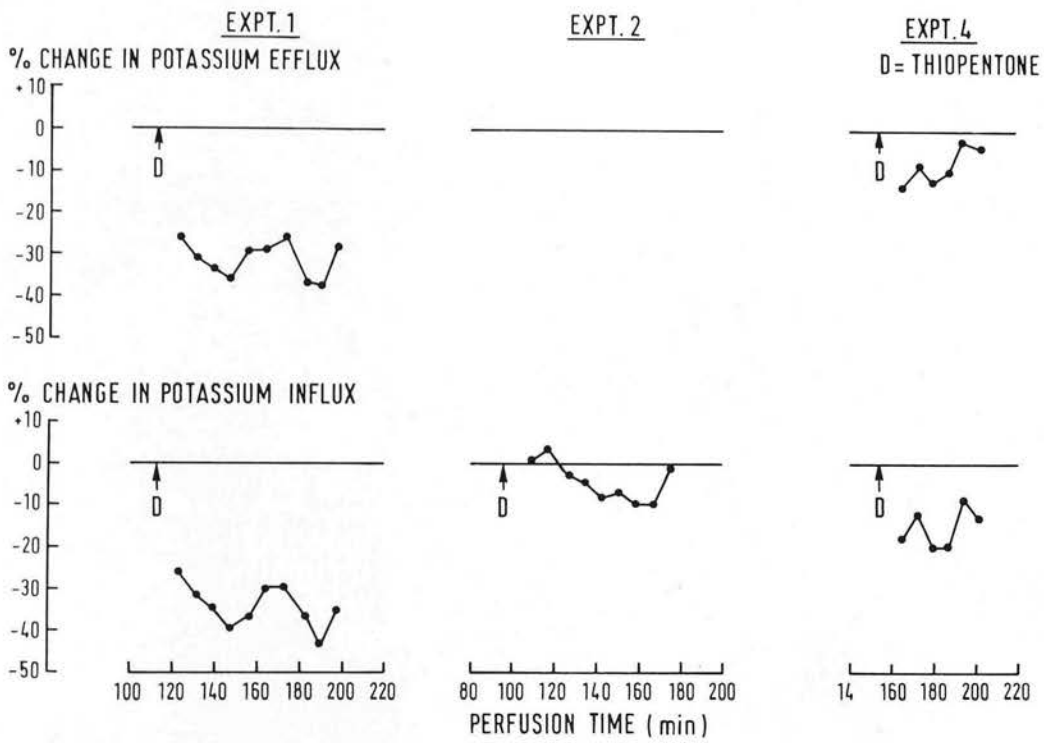


FIG. 2:11 Time course of action of sodium thiopentone on c.s.f. potassium efflux and influx.

At each sampling point the difference between the observed flux value and the extrapolated pre-drug control flux value (see text pages 148 to 155) has been expressed as a percentage of the mean flux value in the pre-drug control period.

($p < 0.05$) decrease of 3.8% in the potassium influx. Examination of the time course of the drug action in this experiment (Fig. 2:11, Expt. 2) suggests a more delayed effect.

In none of these experiments was there a significant decrease in rectal temperature during the period of anaesthesia.

Sodium Pentobarbitone : Effect on C.S.F. Potassium Fluxes

Sodium pentobarbitone (60 mg/ml solution) was administered intravenously in a dose which was judged to give a level of anaesthesia comparable with that achieved in the thiopentone experiments. The induction period with sodium pentobarbitone was appreciably slower (3-8 minutes) than with thiopentone but in fact the dosage used was very similar (23 mg/kg). In one experiment (experiment 8, Fig. 2:12) the initial dose administered was insufficient and a supplementary dose of 3 mg/kg was administered to restore the desired level of anaesthesia.

The potassium efflux data from three sodium pentobarbitone experiments are shown in Table 2:8. The time courses of significant drug effects are shown in Fig. 2:12. In all three experiments the anaesthetic caused a significant decrease in the potassium efflux from the c.s.f. The 42% decrease seen in experiment 7 is comparable to the 31.6% decrease seen in an earlier sodium thiopentone experiment (experiment 1, Table 2:6). The decreases in the potassium efflux seen in experiments 6 and 8 were both much smaller (7.3% and 3.1% respectively).

TABLE 2:8 Efflux of Potassium from the C.S.F. : Effect of Pentobarbitone Anaesthesia

Expt.	Date	Dog	Potassium Efflux Pre-Drug (meq/min)	Observed Post-Drug Potassium Efflux as % of Pre-Drug Value	Change in[2] Potassium Efflux due to drug (meq/min)	% Change in[3] Potassium Efflux due to drug	Effect of drug on efflux	t-value[4]	Significance of drug effect
6	20/4/71	A 7	0.8608 ± 0.0525(9)[1]	84.9 ± 6.4%(15)	-0.0633 ± 0.0338(15)	7.3%	↓	7.00	< 0.001
7	28/4/71	A 1	0.7621 ± 0.0416(7)	54.0 ± 15.6%(9)	-0.3211 ± 0.0281(9)	42%	↓	32.28	< 0.001
8	4/8/71	A11	0.5430 ± 0.0285(13)	87.8 ± 4.4%(10)	-0.0168 ± 0.0130(10)	3.1%	↓	3.82	< 0.01

Footnotes - See Table 2:

TABLE 2: 9 : Influx of Potassium from the C.S.F. : Effect of Sodium Pentobarbitone Anaesthesia

Expt.	Date	Dog	Potassium Influx Pre-Drug (meq/min)	Observed Post-Drug Potassium Influx as % of Pre-Drug Value	Change in[2] Potassium Influx due to drug (meq/min)	% Change in[3] Potassium Influx due to drug	Effect of drug on influx	4 t-value	Significance of drug effect
6	20/4/71	A 7	0.9624 ± 0.0564(9)[1]	84.1 ± 5.4%(15)	-0.1176 ± 0.0412(15)	12.2%	↓	10.67	<0.001
7	28/4/71	A 1	0.7520 ± 0.0474(7)	50.7 ± 14.4%(9)	-0.3893 ± 0.0389(9)	49.7%	↓	28.34	<0.001
8	4/8/71	A11	0.7198 ± 0.0627	85.3 ± 7.7%(10)	-0.0745 ± 0.0473(10)	10.4%	↓	4.72	<0.01

Footnotes - See Table 2:

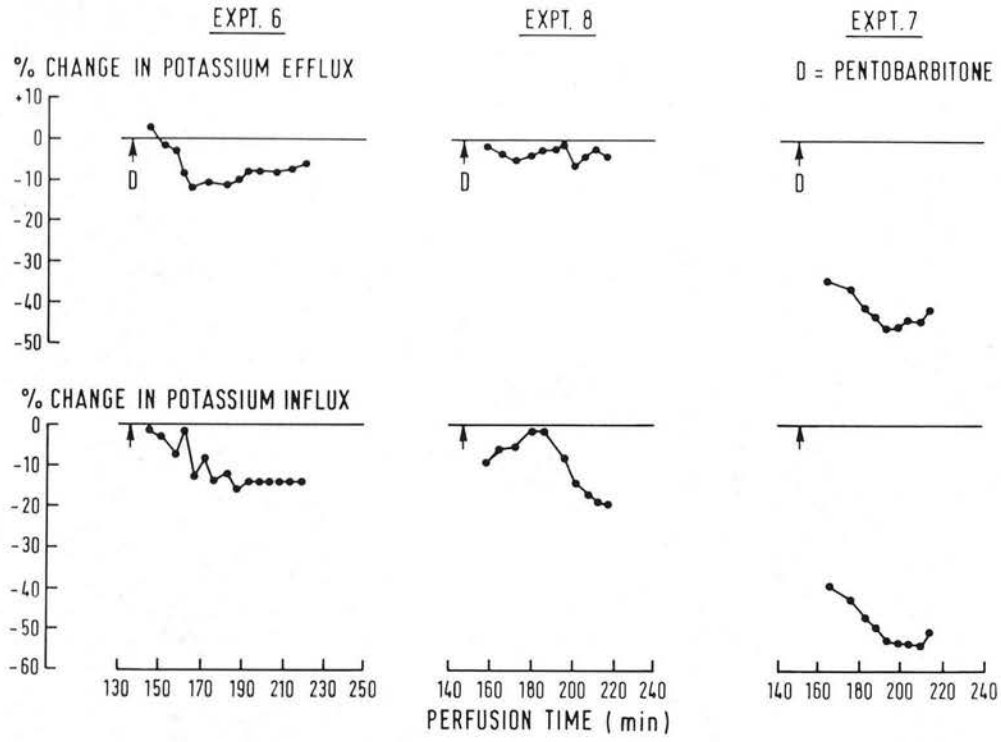


FIG. 2:12 Time course of action of sodium pentobarbitone on c.s.f. potassium efflux and influx.

At each sampling point the difference between the observed flux value and the extrapolated pre-drug control flux value (see text pages 148 to 155) has been expressed as a percentage of the mean flux value in the pre-drug control period.

The potassium influx data from these three experiments are shown in Table 2:9. As is the case with thiopentone anaesthesia it appears that pentobarbitone anaesthesia has a greater depressant effect on potassium influx than on efflux. The largest change was seen in experiment 7 where the drug decreased the potassium influx by 49.7%. The decreases in experiments 6 and 8 were 12.2% and 10.4% respectively.

In keeping with the gradual onset of anaesthesia, the maximum changes in c.s.f. potassium fluxes were not observed until some 30 or 40 minutes after administering the drug (Fig. 2:12).

As in the thiopentone anaesthesia experiments, no significant change in the dog's body temperature was noted during the period of pentobarbitone anaesthesia.

Diazepam : Effect on C.S.F. Potassium Fluxes

Diazepam (5 mg/ml solution) was administered by slow intravenous injection. The dose which was given, namely 20 mg, is twice the initial dose which is used in man to induce brief anaesthesia or control status epilepticus. Before the injection was completed the dog usually displayed signs of muscle relaxation and became slightly ataxic, but anaesthesia was not induced. While the dogs appeared drowsy, their eyes remained open and they responded, in as normal a fashion as their ataxia allowed, to stimuli such as loud noises.

The potassium efflux data from three diazepam experiments is shown in Table 2:10. In two of the experiments (experiments

TABLE 2:10 : Efflux of Potassium from the C.S.F. : Effect of Diazepam

Expt.	Date	Dog	Potassium Efflux Pre-Drug (meq/min)	Observed Post-Drug Potassium Efflux as % of Pre-Drug Value	Change in Potassium Efflux due to drug (meq/min)	% Change in Potassium Efflux due to drug	Effect of drug on efflux	t-value	Significance of drug effect
9	21/4/69	A 2	0.5696 ± 0.0139(6)	82.9 ± 7.7%(8)	-0.0562 ± 0.0402 (8)	9.9%	↓	3.70	< 0.01
11	28/9/71	A 9	0.6076 ± 0.0089(8)	93.3 ± 4.9%(11)	-0.0129 ± 0.0197 (11)	2.1%	↓	2.06	-
12	4/11/71	A 9	0.5013 ± 0.0192(9)	85.6 ± 8.0%(9)	-0.0486 ± 0.0302 (9)	9.7%	↓	4.82	< 0.01

Footnotes - See Table 2:

TABLE 2: 11 : Influx of Potassium from the C.S.F. : Effect of Diazepam

Expt.	Date	Dog	Potassium Influx Pre-Drug (meq/min)	Observed Post-Drug Potassium Influx as % of Pre-Drug Value	Change in[2] Potassium Influx due to drug (meq/min)	% Change in[3] Potassium Influx due to drug	Effect of drug on influx	4 t-value	Significance of drug effect
9	21/4/69	A 2	0.5395 ± 0.0230[1](6)	84.6 ± 5.4%(8)	-0.0603 ± 0.0356(8)	11.2%	↓	4.48	< 0.01
11	28/9/71	A 9	0.5930 ± 0.0242(8)	93.7 ± 5.9%(11)	-0.0282 ± 0.0307(11)	4.8%	↓	2.94	< 0.02
12	4/11/71	A 9	0.6291 ± 0.0217(9)	90.9 ± 6.5%(10)	-0.0379 ± 0.0351(10)	6.0%	↓	3.24	< 0.02

Footnotes - See Table 2:

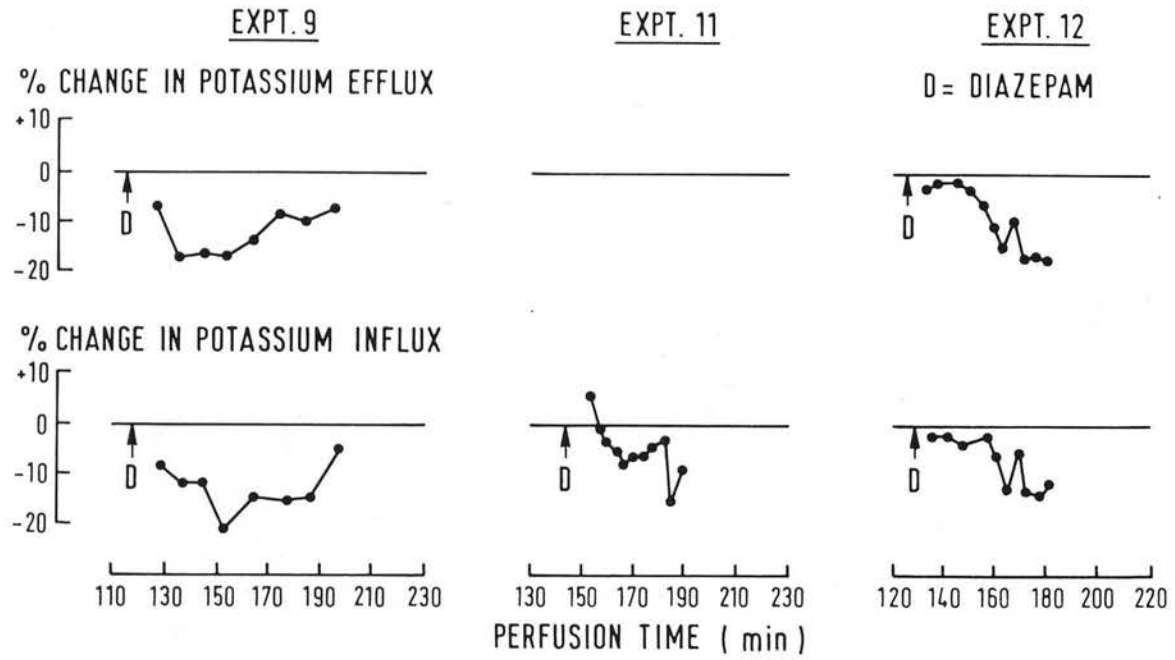


FIG. 2:13 Time course of action of diazepam on c.s.f. potassium efflux and influx.

At each sampling point the difference between the observed flux value and the extrapolated pre-drug control flux value (see text pages 148 to 155) has been expressed as a percentage of the mean flux value in the pre-drug control period.

9 and 12) diazepam caused a 9-10% decrease in the potassium efflux from the c.s.f. A decreased efflux was also seen in experiment 11 but this was not significant ($p > 0.05$).

The potassium influx data is shown in Table 2:11. In experiment 9 there was a significant ($p < 0.01$) decrease in the potassium influx due to diazepam, but in experiments 11 and 12 the decreases seen were smaller at 4.8% and 6.0% respectively. In both experiments 11 and 12 there appeared to be a time lag before the drug had any appreciable effect on the potassium fluxes (Fig. 2:13). Even in experiment 9 the peak effect was not seen until 25-30 minutes after administering the drug.

This time lag before c.s.f. potassium fluxes were maximally affected contrasted with the behavioural changes observed. In behavioural terms the dog's recovery started within ten minutes of giving the injection and the dogs became progressively more alert and active.

Diphenylhydantoin : Effect on C.S.F. Potassium Fluxes

Diphenylhydantoin was administered intravenously (48 mg/ml solution) in a dose of 80 mg (effectively 5 mg/kg). No behavioural changes or physical signs were seen after giving the drug.

The potassium efflux data from two diphenylhydantoin experiments are shown in Table 2:12. No significant change was seen in either experiment after giving diphenylhydantoin.

The influx data shown in Table 2:13 demonstrate that diphenylhydantoin slightly reduced potassium influx into the c.s.f. in both

TABLE 2: 12 : Efflux of Potassium from the C.S.F. : Effect of Diphenylhydantoin

Expt.	Date	Dog	Potassium Efflux Pre-Drug (meq/min)	Observed Post-Drug Potassium Efflux as % of Pre-Drug Value	Change in Potassium Efflux due to drug (meq/min)	% Change in Potassium Efflux due to drug	Effect of drug on efflux	t-value	Significance of drug effect
14	25/5/71	A 7	0.9300 ± 0.0470(10)	92.5 ± 4.4%(10)	+0.0002 ± 0.0400(10)	0	—	0.02	-
15	29/9/71	A10	0.7021 ± 0.0330(15)	91.4 ± 5.4%(12)	-0.0130 ± 0.0412(12)	1.9%	↓	1.05	-

[1] [2] [3] [4]

Footnotes - See Table 2:

TABLE 2:13 : Influx of Potassium from the C.S.F. : Effect of Diphenylhydantoin

Expt.	Date	Dog	Potassium Influx Pre-Drug (meq/min)	Observed Post-Drug Potassium Influx as % of Pre-Drug Value	Change in[2] Potassium Influx due to drug (meq/min)	% Change in[3] Potassium Influx due to drug	Effect of drug on influx	4 t-value	Significance of drug effect
14	25/5/71	A 7	1.0258 ± 0.0603(10)	93.7 ± 4.0%(10)	-0.0311 ± 0.0399(10)	3%	↓	2.33	< 0.05
15	29/9/71	A10	0.7296 ± 0.0372(15)	89.7 ± 5.9%(12)	-0.0502 ± 0.0416(12)	6.9%	↓	4.00	< 0.01

Footnotes - See Table 2:

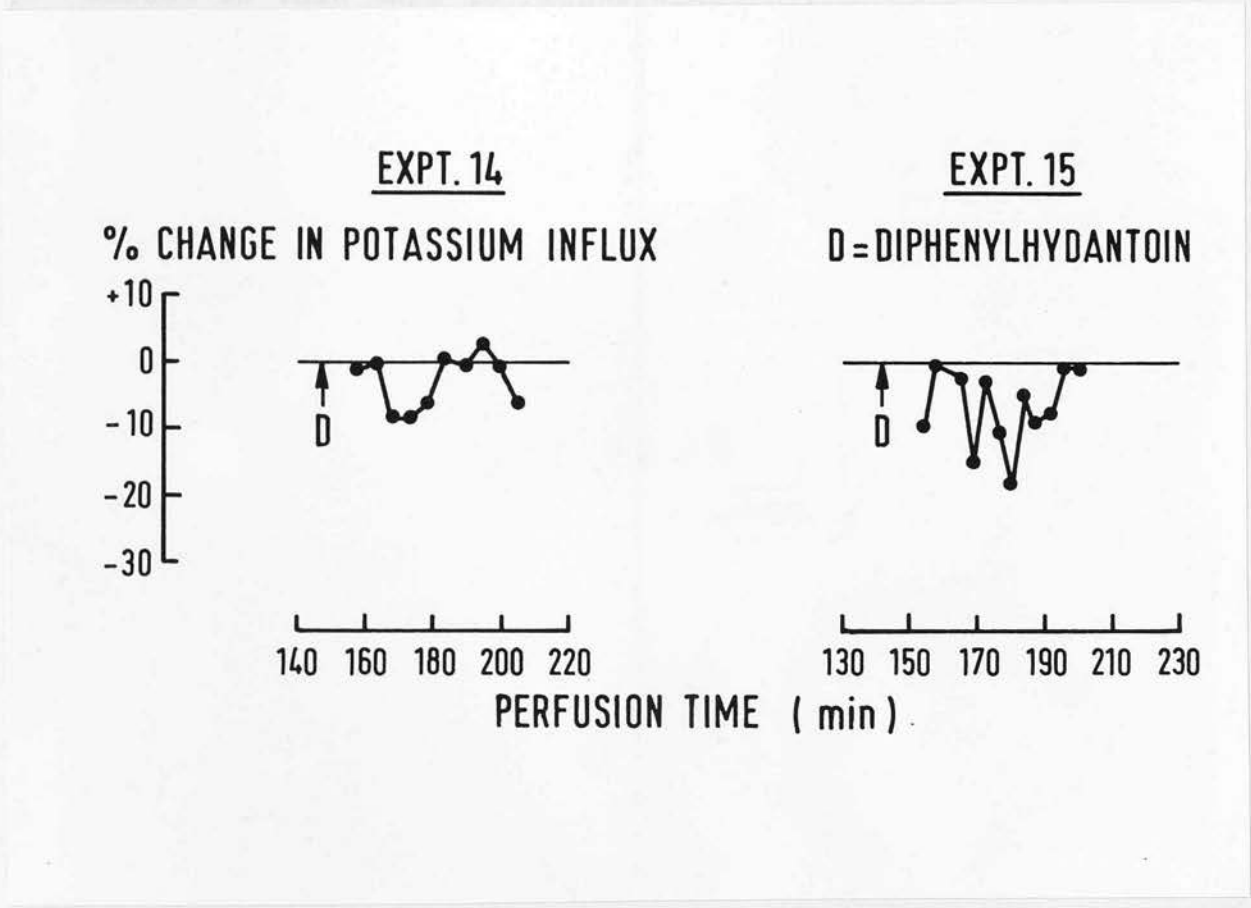


FIG. 2:14 Time course of action of diphenylhydantoin on c.s.f. potassium influx.

At each sampling point the difference between the observed flux value and the extrapolated pre-drug control flux value (see text pages 148 to 155) has been expressed as a percentage of the mean flux value in the pre-drug control period.

experiments. When the time course of this action was plotted as in Fig. 2:14 it was seen that the data from experiment 14 was highly irregular and therefore the significance of the drug effect in this case is rather doubtful.

Paraldehyde : Effect on C.S.F. Potassium Fluxes

As distinct from all the other drugs used in this study paraldehyde was administered by intramuscular injection. The injection was extremely painful and the dose of 4-6 ml was split into 4 aliquots to be administered at separate sites. Over a period of five to fifteen minutes the dogs gradually lapsed into a state of anaesthesia in which they no longer responded even to strong pressure between the toes.

As can be seen from Table 2:14, paraldehyde caused an increase in the efflux of potassium from the c.s.f. (13% in experiment 16; 8.2% in experiment 17) in two of the three experiments. In the third (experiment 18) it had no effect.

Table 2:15 presents the influx data from the same experiments. Only in experiment 16 was there a significant change ($p < 0.01$). Again, the change seen was an increase (9.8%) in the influx.

This drug was the only one of the five studied which at any time caused a positive change in c.s.f. potassium fluxes.

The relatively slow onset of paraldehyde anaesthesia after intramuscular injection was reflected in the slow appearance of effects on c.s.f. potassium fluxes (Fig. 2:15).

TABLE 2:14 Efflux of Potassium from the C.S.F. : Effect of Paraldehyde

Expt.	Date	Dog	Potassium Efflux Pre-Drug (meq/min)	Observed Post-Drug Potassium Efflux as % of Pre-Drug Value	Change in [2] Potassium Efflux due to drug (meq/min)	[3] % Change in Potassium Efflux due to drug	Effect of drug on efflux	[4] t-value	Significance of drug effect
16	18/6/69	A 5	0.6423 ± 0.0303(6) [1]	104.6 ± 5.7%(12)	+0.0828 ± 0.0554(12)	13%	↑	4.96	< 0.001
17	17/2/71	A 1	0.5902 ± 0.0388(9)	96.5 ± 3.3%(14)	+0.0485 ± 0.0307(14)	8.2%	↑	5.68	< 0.01
18	30/9/71	A 7	1.0774 ± 0.0250(14)	95.1 ± 1.5%(15)	-0.0000 ± 0.0242(15)	0	-	-	-

Footnotes - See Table 2:

TABLE 2:15 : Influx of Potassium from the C.S.F. : Effect of Paraldehyde

Expt.	Date	Dog	Potassium Influx Pre-Drug (meq/min(	Observed Post-Drug Potassium Influx as % of Pre-Drug Value	Change in[2] Potassium Influx due to drug (meq/min)	% Change in[3] Potassium Influx due to drug	Effect of drug on influx	4 t-value	Significance of drug effect
16	18/6/69	A5	0.5812 ± 0.0317(6)[1]	105.0 ± 7.8%(12)	+0.0570 ± 0.0458(12)	9.8%	↑	4.12	<0.01
17	17/2/71	A1	0.5785 ± 0.0446(9)	98.4 ± 4.5%(14)	-0.0020 ± 0.0268(14)	0.3%	↓	-0.26	-
18	30/9/71	A7	1.1475 ± 0.0294(14)	96.3 ± 2.6%(15)	-0.0059 ± 0.0333(15)	0.5%	↓	0.67	-

Footnotes - See Table 2:

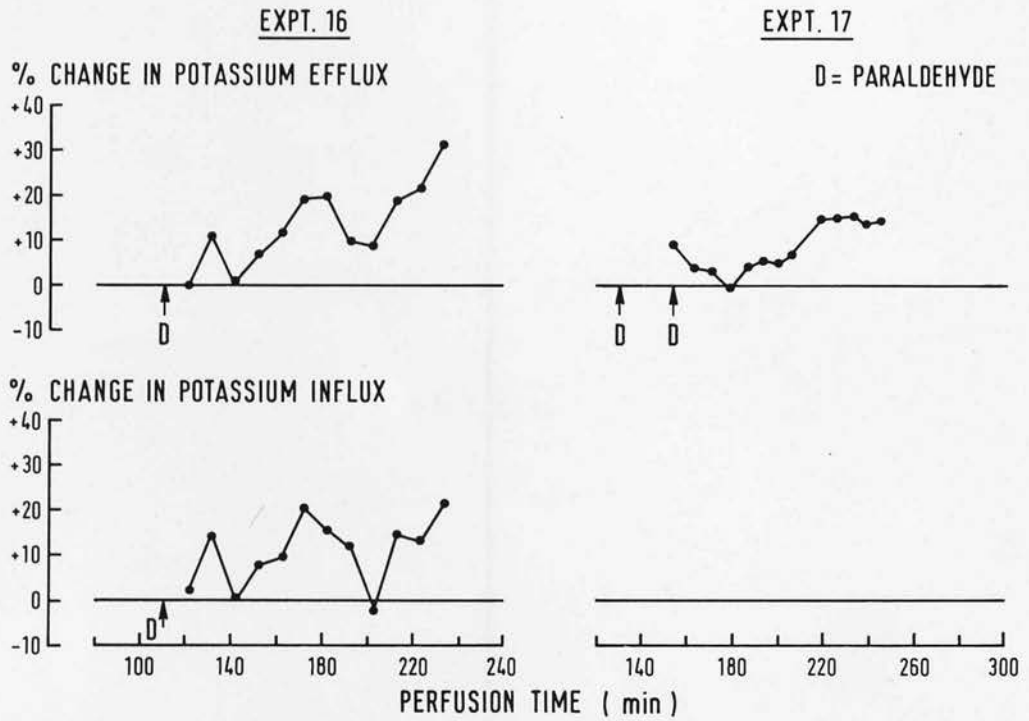


FIG. 2:15 Time course of action of paraldehyde on c.s.f. potassium efflux and influx.

At each sampling point the difference between the observed flux value and the extrapolated pre-drug control flux value (see text pages 148 to 155) has been expressed as a percentage of the mean flux value in the pre-drug control period.

Other factors affecting c.s.f. potassium fluxes

In two experiments (3 and 4) we examined the effects of sodium thiopentone anaesthesia on c.s.f. pH and pCO_2. In experiment 3 we also monitored end-alveolar air pCO_2.

The results are shown in Fig. 2:16. After anaesthesia was induced by thiopentone (Expt. 3) there was an immediate increase in end alveolar pCO_2. In neither of the two experiments was there a significant post-drug alteration in the pCO_2 of the c.s.f. In both experiments there was evidence of a small increase in the pH of the c.s.f. (less than 0.1 pH unit).

In certain preliminary experiments in addition to measuring the c.s.f. fluxes of potassium we simultaneously measured the c.s.f. fluxes of sodium and calcium, using ^{22}Na and ^{45}Ca. The fluxes of these two cations were not significantly affected by barbiturate anaesthetics despite the alterations caused in potassium fluxes.

Experiments not included in the analysis of data

As a result of a high level of fluctuation in flux values between adjacent "steady-state" values many of the results from early perfusion experiments have not been fully analysed. The high error variation in these experiments was caused by several factors.

During the development of the technique a reliable fraction collector was not available and perfusate samples had to be

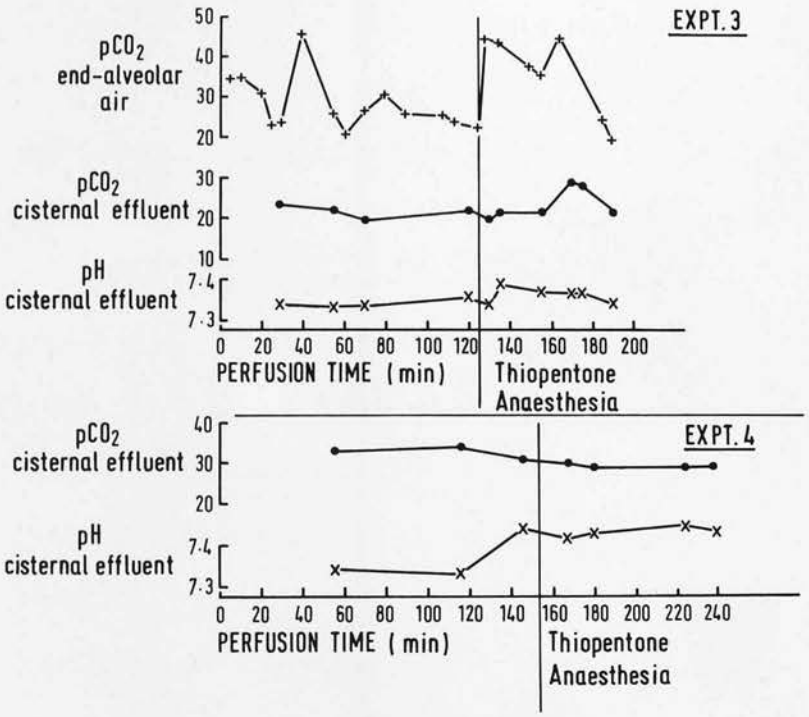


FIG. 2:16 Effect of sodium thiopentone anaesthesia on c.s.f. pH and pCO_2, and on end-alveolar air pCO_2. pCO_2 figures are shown in torr.

weighed and carefully checked as to the length of time of collection. Another problem encountered initially was in performing satisfactory inulin and potassium assays on samples which unavoidably had to be stored for a few days prior to assay.

Perhaps the commonest problem was that of achieving satisfactory perfusion in a fully conscious and often highly active dog. On many occasions a perfusion had to be stopped to allow repositioning of the cisternal needle or enable a blockage to be cleared from the system. These difficulties were largely overcome by selecting only dogs of a docile temperament for perfusion experiments and by paying meticulous attention to the length and sharpness of the perfusion needles used for a particular dog.

Although these early experiments have not been considered satisfactory in the light of our results with established techniques they indicated qualitatively that sodium pentobarbitone and sodium thiopentone reduced c.s.f. potassium fluxes.

Two experiments (10 and 13) shown in Appendix I have not been included in the results section. In these experiments the perfusions appeared to go normally and the accuracy of the analytical results was confirmed by repeating the biochemical assays. As can be seen however, the inulin clearance in both experiments was extremely high and for this reason the data have not been considered reliable. Both these experiments concerned the same dog but this dog was used for other experiments, before,

between and after the two excluded experiments and on these occasions a normal inulin clearance was always observed. The values for potassium efflux and influx (which are of course corrected for loss of potassium in fluid absorbed in bulk into the subarachnoid spaces) in these two experiments did not appear abnormal and so the experiments have been included with the others in Appendix I. It is interesting that the diphenylhydantoin experiment (Expt. 13) agrees with the other two diphenylhydantoin experiments (14 and 15) in that the drug appeared to decrease the influx of potassium into the c.s.f.

DISCUSSION

^{42}K efflux from the c.s.f. as measured in these experiments is a composite parameter comprising efflux into brain and efflux into blood. Although the relative contribution of each efflux pathway has not been experimentally determined some information on this subject can be derived from previous ventricular-cisternal perfusion studies of potassium fluxes in anaesthetised animals.

In studies on the anaesthetised dog (Cserr, 1965) rabbit (Bradbury and Davson, 1965) and cat (Katzman et al., 1965) it was found that most of the ^{42}K leaving the c.s.f. could subsequently be recovered from the brain. It appeared that this uptake was governed by simple diffusion and isotopic exchange with the large stores of intracellular potassium in the brain. Such a process seems unlikely to be involved in the long term regulation of c.s.f. potassium.

The other major component of potassium efflux from the c.s.f. is potassium transport from the c.s.f. into blood. This efflux mechanism has been characterised by the studies of Bradbury and Štulcová (1970) on the anaesthetised rabbit. They found that with c.s.f. potassium concentrations below 3.2 meq/l the potassium efflux into blood was responsible for only a small fraction of the total efflux from c.s.f. Above this concentration, the transport of potassium from the c.s.f. into blood increased rapidly, until at a c.s.f. potassium concentration of 10 meq/l

it constituted almost 40% of the total efflux from c.s.f. This efflux from c.s.f. to blood was inhibited by ouabain. Bradbury and Šťulcová hypothesised the existence of a sodium-potassium pump mechanism (potassium directed towards the blood) situated either at the choroid plexus or at the capillary blood-brain barrier. The existence of such a pump in the choroid plexus seems doubtful since Wright (1970) found no net flux of potassium across the choroid plexus of the frog nor were the individual one-way potassium fluxes across this tissue inhibited by ouabain. However the findings of a recent study by Bradbury, Segal and Wilson (1972) indicate that such a pump may be located at the blood-brain interface of cerebral capillaries. The characteristics of the mechanism for transporting potassium from c.s.f. into blood suggest that it may be a vital factor in the homeostasis of c.s.f. and brain extracellular fluid potassium (Bradbury and Šťulcová, 1970).

How do these findings in the anaesthetised animal relate to our own findings in the conscious animal? Since the pump transporting potassium from the c.s.f. to the blood appears to be concentration dependent, and not very active at normal levels of c.s.f. potassium, it is unlikely to have been responsible for more than 20% of c.s.f. potassium efflux seen in our conscious animals. Thus, in our experiments, at least 80% of the observed efflux of ^{42}K from the c.s.f. was probably due to isotopic exchange with unlabelled potassium in the brain. As a corollary

it seems likely that the major portion of the potassium influx into c.s.f. was derived from brain. This would agree with the evidence that ^{42}K in the plasma penetrates very slowly into c.s.f., other than by way of newly secreted fluid (Cserr, 1965; Bradbury and Davson, 1965; Katzman et al. 1965).

We have found that the barbiturate anaesthetics, sodium thiopentone and sodium pentobarbitone, in a comparatively low dosage (23 mg/kg) may depress the potassium fluxes of c.s.f. In view of this fact it seems possible that previous estimates of c.s.f. potassium fluxes in anaesthetised animals (30 mg/kg pentobarbitone was used in the studies of Bradbury and Štulcová) may not be representative of the fluxes in the conscious animal. The question arises as to which component of the fluxes has been affected by the anaesthetics. Two facts suggest that the anaesthetics may affect the potassium exchange with the brain.

In the first place, in the deeply anaesthetised animals studied by Bradbury and Štulcová (1970) there did not appear to be any loss of homeostatic control of potassium in the c.s.f. This suggests that the pump transporting potassium from the c.s.f. into the blood is unaffected by anaesthetics.

In the second place, in our experiments we have observed decreases of up to 40% in the total efflux of potassium from the c.s.f. after administering an anaesthetic. Such alterations in efflux are unlikely to have been due to inhibition of the c.s.f. to blood transport since this mechanism probably accounts for

only a small fraction of the total potassium efflux from c.s.f. (Bradbury and Stulcová, 1970).

For these two reasons it seems probable that the barbiturate anaesthetics were depressing the efflux of potassium from the c.s.f. by inhibiting the exchange between potassium in the c.s.f. and potassium in the brain. This would fit with the depressant effects of anaesthetics on neuronal activity since a lesser number of excitation events across nerve membranes would be expected to lead to less rapid mixing of intra- and extracellular potassium. In this context it has been suggested by Thesleff (1956) that pentobarbitone may decrease the resting potassium conductance across excitable membranes. Pentobarbitone has also been shown to be antagonistic to the action of potassium on atrial muscle fibres (Smith and Gershwin, 1969).

A depressant effect of the barbiturates on potassium exchange across neuronal membranes, such as we have postulated here, might be expected to decrease not only potassium efflux from, but also potassium influx into the c.s.f. In fact this is exactly what was seen in our experiments, the influx being depressed in a parallel fashion to the efflux. The actual potassium concentration in the c.s.f. remained unaltered.

This lack of effect of pentobarbitone anaesthesia on the level of potassium in the c.s.f. contrasts markedly with its depressant effect on the plasma potassium (Bradbury and Davson, 1965), providing further proof that pentobarbitone anaesthesia

does not affect those transport mechanisms which are responsible for the homeostasis of c.s.f. potassium.

The variability of the anaesthetic effects is not easy to explain. An explanation based on between-dog variation is not tenable since we observed very different depressions of c.s.f. potassium fluxes on different occasions in the same dog. We have considered the possibility that the changes in c.s.f. potassium fluxes were secondary to anaesthetic-induced changes in the electrical potential between c.s.f. and blood. Held, Fencl and Pappenheimer (1964) have shown that this potential in the conscious goat is approximately 6.8 mv (c.s.f. positive). In dogs under pentobarbitone anaesthesic they recorded potentials varying between -2 and 7 mv. They ascribed this large spread of values to variationsin arterial pH. In two of our own perfusion experiments in which sodium thiopentone was administered we recorded the pH and pCO_2 of the cisternal effluent. In one of these experiments the pCO_2 of end alveolar air was also recorded. The results suggested that the barbiturate anaesthetics were not seriously affecting c.s.f. pH or pCO_2. It therefore seems unlikely that changes in the c.s.f. blood potential were responsible for the observed alterations in c.s.f. potassium fluxes.

From preliminary experiments in which sodium and calcium fluxes from the c.s.f. were measured in parallel with potassium fluxes, it would appear that barbiturate anaesthesia does not significantly alter sodium and calcium fluxes, even when it produces

depressions in c.s.f. potassium fluxes.

Perhaps the explanation for the variability of the anaesthetic effect may lie in differences in the degree of anaesthesia from experiment to experiment. If this is true however, it must be that barely observable changes in depth of anaesthesia can produce large changes in c.s.f. potassium fluxes.

The alterations in c.s.f. potassium fluxes produced by the three anticonvulsants studied were near the sensitivity limits of the method. For this reason any interpretation of their actions must be tentative.

Diazepam produced a slight decrease in both efflux and influx of potassium. The fact that, as with the barbiturate anaesthetics, efflux and influx were equally affected suggested that the drug might be inhibiting a single exchange mechanism affecting both influx into and efflux out of the c.s.f. The likeliest mechanism would seem to be the exchange process between brain intracellular and extracellular potassium. However, unlike the barbiturates, diazepam did not produce anaesthesia so it is possible that a different mechanism of action may have been responsible for the decreased potassium exchange between c.s.f. and brain.

As yet no explanation is available for diazepam's anti-convulsant action even though it is very widely used in the control of status epilepticus. It is worth noting that there may be a marked species difference in the effect of this drug since the dose administered to the dogs in our experiments (20 mg) would

have been sufficient, in absolute terms, to anaesthetise a man five times the dog's weight.

Diphenylhydantoin had no effect on potassium efflux from the c.s.f. but there was evidence that it had a slight depressant effect on potassium influx. These findings contrast with the report by Woodbury and Kemp (1971) that diphenylhydantoin increases potassium efflux from the c.s.f. However these results are not so divergent as might at first sight appear, since both bring about a lowered c.s.f. potassium concentration. In our experiments we only measured total efflux of potassium from the c.s.f. and it is possible that the efflux from c.s.f. to blood could have been increased and the efflux of potassium into brain decreased by diphenylhydantoin. We would not have noticed such an effect since the total efflux from c.s.f. would have been unchanged. If the potassium influx from brain was also depressed these findings would be consistent with a dual action of diphenylhydantoin, firstly in reducing potassium exchange across the neuronal membrane and secondly in increasing potassium efflux from the c.s.f. to the blood. If, as seems probable, Woodbury and Kemp (1971) used anaesthetised animals in their study then the former action, namely decreasing potassium exchange across the neuronal membrane, may have been obscured by a similar action of the anaesthetic. In this case they would only have observed an increased efflux of potassium from the c.s.f. In such circumstances our own findings and those of Woodbury and Kemp (1971) would

be compatible. However the difference between the two studies may be due to a number of factors such as the species of animal being studied; the dosage of diphenylhydantoin used; whether the animal was being treated chronically or acutely; the method used to investigate potassium efflux from the c.s.f.

Woodbury and Kemp (1971) have hypothesised that the anti-convulsant action of diphenylhydantoin is due to its stabilising effect on excitable membranes by increasing sodium transport out of the cell, thus producing a hyperpolarised membrane. This is supported by their own finding of a decreased intracellular sodium content and Festoff and Appel's (1968) finding that diphenylhydantoin stimulates sodium-potassium ATPase activity. However Izquierdo and Nasello (1970) have suggested that diphenylhydantoin may antagonise post-tetanic potentiation and seizure activity by an action on potassium transport. It may therefore be premature to exclude an effect on potassium in the brain from the action of diphenylhydantoin.

Paraldehyde was unique among the drugs studied in that it appeared to increase potassium efflux from the c.s.f. in two out of three cases. In one of these two cases the influx was not affected but in the other it also was increased. The stimulation of c.s.f. potassium fluxes contrasted with the drug's obvious anaesthetic action. The drug was therefore similar to the barbiturates in terms of anaesthetic effects but dissimilar in its effects on c.s.f. potassium fluxes. This was surprising

considering that Thesleff (1956) has reported that these drugs have very similar effects on the ionic permeability of muscle excitable membrane. However very little is known about the mechanism of action of paraldehyde. It may be that we were observing a stimulant effect on potassium fluxes due to an independent action of the drug or it may be that some intermediary, such as the drug's metabolite acetaldehyde, was affecting potassium fluxes.

In conclusion then how has our study contributed to work in this field?

From a technical aspect it would appear that the animal techniques and analytical treatment of results which we have evolved provide, for the first time, a method of monitoring c.s.f. potassium fluxes in the fully conscious and free-moving animal over a period of 2 to 3 hours. The technique would appear to have a limited application in the study of transport of potassium from the c.s.f. to the blood since this can only be accurately measured by examining the amount of ^{42}K left in the brain after perfusion. It would seem more economic to develop this type of study in the rabbit, using the conscious perfusion technique evolved by Moir and Dow (1970).

With respect to drug studies we have shown that sodium pentobarbitone, sodium thiopentone and diazepam can all depress the c.s.f. potassium fluxes both into and out of c.s.f. We have speculated that these changes reflect a decrease in the exchange

of potassium between brain intra- and extracellular compartments.

There is some evidence that paraldehyde stimulates potassium exchange between c.s.f. and the brain (at the same time as it produces anaesthesia), and that diphenylhydantoin may decrease the influx of potassium into the c.s.f.

It would therefore appear from these studies that decreases in potassium fluxes of the c.s.f. may be associated with anaesthetic or anticonvulsant action but it would seem, in the case of paraldehyde at least, that it is possible to achieve both these pharmacological actions without any alteration in c.s.f. potassium fluxes.

SUMMARY

A new technique was developed for "open" perfusion of the cerebroventricular system of the conscious dog. A kinetic analysis of the perfusion system is described. Dogs were perfused from lateral ventricle to cisterna magna with artificial c.s.f. to which had been added a non-diffusible marker substance, inulin, and tracer amounts of ^{42}K. Inulin, total potassium and ^{42}K estimations on samples of inflow and outflow fluid allowed calculation of both potassium efflux from, and potassium influx into, the c.s.f.

The effect of barbiturate anaesthetics and anticonvulsants on these parameters has been studied.

It was found that:-

1) sodium thiopentone and sodium pentobarbitone, in a dose sufficient to induce light anaesthesia, may depress both potassium efflux from, and potassium influx into, the c.s.f.

2) diazepam has consistent depressant effect on the potassium fluxes of c.s.f. The effect is less marked than that seen with the barbiturates.

3) diphenylhydantoin has no effect on potassium efflux from the c.s.f. but may slightly depress potassium influx into the c.s.f.

4) paraldehyde, in a dose sufficient to induce light anaesthesia, slightly increases the potassium fluxes of c.s.f.

The depressant effect of the barbiturates and diazepam on c.s.f. potassium fluxes has been interpreted as being secondary to a depressant action on potassium exchange across neuronal membranes in the brain.

These findings are discussed in relation to:-

1) previous studies on c.s.f. potassium fluxes.

2) possible mechanisms of actions of the drugs.

It was concluded that the anaesthetic or anticonvulsant action of a drug is not related to the drug's effect on c.s.f. potassium fluxes.

SECTION III

A NEW MICROTECHNIQUE FOR THE QUANTITATIVE DETERMINATION OF FREE AMINO ACIDS IN BRAIN TISSUE: ITS APPLICATION TO A STUDY OF GABA AND OTHER AMINO ACIDS IN A MODEL EPILEPTIC FOCUS.

INTRODUCTION

Since the first reports of the presence of significant amounts of δ-amino butyric acid (GABA) in the brain (Awapara, Landua, Fuerst and Seale, 1950; Roberts and Frankel, 1950) there has been much evidence to suggest that this substance may have an inhibitory transmitter function in the central nervous system, and that disorders in GABA metabolism may be causally related to convulsive phenomena.

Krnjević (1970) has recently carried out a comprehensive review of the evidence for GABA's transmitter function in the brain and in this short introduction I shall only summarise the main points in the argument.

From Florey's early work on the depressant effects of beef brain extracts on the crayfish stretch receptor (Florey, 1954) it emerged that the active principle, which had been named Factor I by Florey, was in fact GABA (Bazemore, Elliot and Florey, 1957). On the same tissue Kuffler and Edwards (1958) and Takeuchi and Takeuchi (1965) established the identical effects of synaptic inhibition and iontophoretic application of GABA. As a final confirmation of GABA's transmitter role it was demonstrated by Otsuka, Iversen, Hall and Kravitz (1965) that stimulation of the inhibitory nerve fibres of this preparation caused a specific release of GABA.

The evidence is not so clear-cut in the mammalian central nervous system,but the early reports of Hayashi (1956);

Purpura, Girado, Smith, Callan and Grundfest (1959); Curtis and Watkins (1960); Krnjević and Phillis (1963) and Crawford and Curtis (1964) established the depressant effects of GABA on certain neurones in the mammalian central nervous system. It is now known that the post-synaptic hyperpolarisations produced by both synaptic inhibition and the application of GABA have an identical reversal potential (Krnjević and Schwartz, 1967; Dreifus, Kelly and Krnjević, 1969) and that both are almost certainly due to an increase in chloride permeability of the post synaptic membrane (Kelly, Krnjević, Morris and Yim, 1969).

Complete electrophysiological investigation of a specific inhibitor pathway by Obata, Ito, Ochi and Sato (1967) gave strong evidence for believing that GABA may be the inhibitory transmitter released onto the cells of Deiter's nucleus by cerebellar Purkinje fibres.

From the many biochemical studies of the distribution of GABA within the brain (Baxter and Roberts, 1959, 1960; Singh and Malhotra, 1962; Kržalić Mandić and Milhailovic 1962; Lovell, Elliot and Elliot, 1963; Fahn and Côté, 1967) we know that like other postulated neurotransmitters such as acetylcholine and noradrenaline, GABA is non-uniformly distributed throughout the grey matter of the brain. We also know that the enzyme which is responsible for the formation of GABA from glutamate (Fig. 3:1), namely glutamate decarboxylase (GAD), is distributed in a very similar pattern throughout the brain (Albers and Brady, 1959;

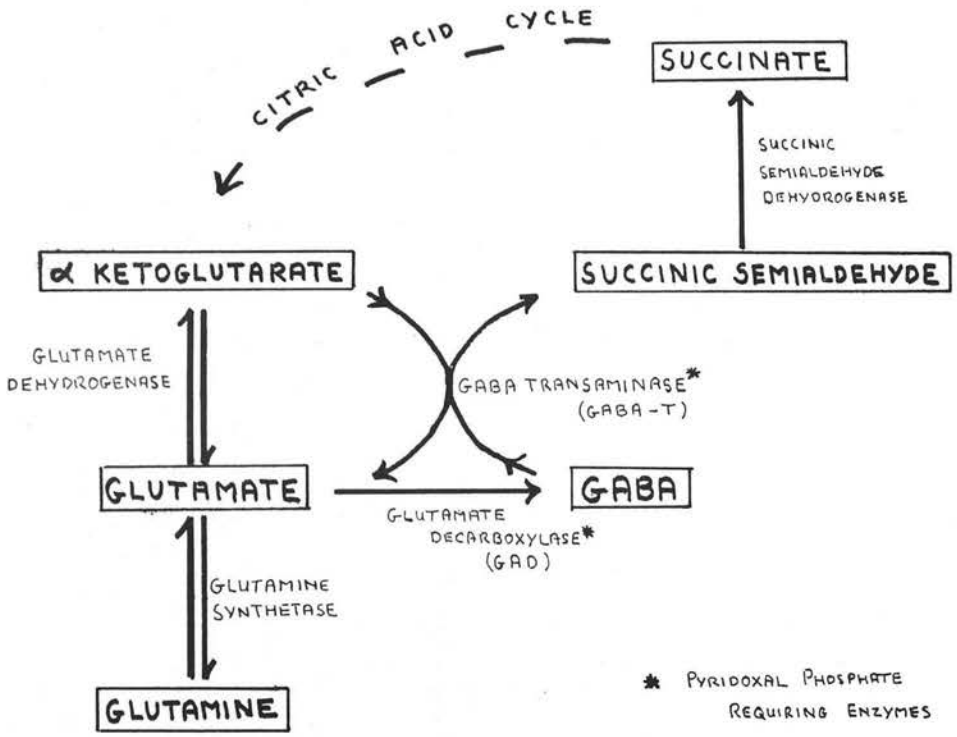


FIG. 3:1 Pathways of metabolism of glutamate and GABA.

Lowe, Robbins and Eyreman, 1958; Müller and Langemann, 1962; Roberts and Eidelberg, 1960; Chalmers, McGeer, Wilson and McGeer, 1970). The concentration of GABA in a particular brain area appears to be directly related to the GAD activity within that area. In contrast the enzyme responsible for the degradation of GABA, namely GABA transaminase (Fig. 3:1), is distributed more generally throughout the central nervous system (Waksman, Rubinstein, Kuriyama and Roberts, 1968). GABA transaminase catalyses the reversible transamination of GABA with α keto-glutarate forming succinic semialdehyde and glutamic acid. Succinic semi-aldehyde is in turn oxidised to succinic acid which then enters the tricarboxylic acid cycle. In the mammalian brain therefore there exist the necessary enzymic mechanisms to synthesise and degrade GABA.

Further support for a transmitter role for GABA can be gained from the presence of specific uptake mechanisms for the transport of GABA into brain cells (Elliot and Van Gelder, 1958; Tsukada, Nagata, Hirano and Matsutami, 1963) and, more specifically, nerve endings (Weinstein, Roberts and Kakefuda, 1963; Neal and Iversen, 1969; Bloom and Iversen 1971; Logan and Snyder, 1971). "In vitro" preparations of cerebral nerve endings have also been shown to contain both GABA (Krnjevic and Whittaker, 1965; Mangan and Whittaker, 1966; Bradford and Thomas, 1969; Kuhar and Snyder, 1970) and its synthesising enzyme, GAD (Salganicoff and De Robertis, 1965; Fonnum, 1968).

The physiological release of GABA has not been easily demonstrated but Katz, Chase and Kopin (1969) and Srinavasan, Neal and Mitchell (1969) have reported that brain slices release GABA when stimulated either electrically, or by depolarising concentrations of potassium. In the "in vivo" situation, Obata and Takeda (1969) have observed an increased release of GABA into the 4th ventricle when the inhibitory pathway from the cerebellum to the nuclei around the ventricle was stimulated. Mitchell and Srinavasan (1969) also found an increased efflux of GABA from the cortex during periods of induced cortical inhibition.

As a final piece of evidence, it has been shown that at some neurones in the mammalian central nervous system both normal synaptic inhibition and the inhibition brought about by the iontophoretic application of GABA are blocked by bicuculline. (Curtis, Duggan, Felix and Johnstone, 1970a, b; Curtis, Duggan and Felix, 1970; Tebecis, Hősli and Haas 1971; Kelly and Reynaud, 1971). However Krnjević has stressed the fact that bicuculline is not a specific antagonist at all inhibitory sites in the central nervous system.

On the basis of all these findings it is reasonable to suggest that GABA may be an inhibitory transmitter in the mammalian central nervous system and as such it may play a part in maintaining the central neuronal population at a "normal" level of excitation. The latter hypothesis has been supported

in a more direct, though perhaps less exacting manner, by many experiments in which the tissue concentrations of GABA have been manipulated by the administration of various drugs.

Kopeloff and Chusio (1965) reported that intravenous or intraperitoneal GABA protected against drug induced convulsions in dogs, while Kobrin and Seifter (1966) noted that intravenous GABA blocked pentylenetetrazole convulsions in day-old chicks. GABA has been shown to prevent the discharge of a chronic epileptic focus caused by either a freezing lesion (Berl, Purpura, Girado and Waelsch, 1959; Berl, Takagaki, and Purpura 1961; Strasberg, Krnjević, Schwartz and Elliot, 1967) or an aluminium oxide implant (Guerrero-Figueroa, De Balbian Verster, Barras and Heath, 1964). GABA has also been shown to protect mice against audiogenic seizures either after topical application to the cortex (Ballantine, 1963), or after systemic administration (Pasquini, Salamone and Gomez, 1968).

Evidence linking GABA with convulsive phenomena has also come from studies in which seizure threshold has been altered by drugs which affect the metabolism of GABA. The enzymes responsible for the synthesis and degradation of GABA, namely glutamate decarboxylase (GAD) and GABA transaminase (GABA-T) respectively, are both dependent on pyridoxal phosphate. This coenzyme is formed from pyridoxal and adenosine tri-phosphate by the enzyme pyridoxal kinase. GAD is particularly sensitive to drugs which reduce the level of free pyridoxal phosphate. Many hydrazines

and hydrazides are capable of forming a covalent bond with the carbonyl group of pyridoxal phosphate, thus reducing its availability for coenzyme functions. Certain hydrazones formed by the reaction between pyridoxal phosphate and a hydrazide may also reduce free levels of pyridoxal phosphate by inhibiting the enzyme pyridoxal kinase (Tapia, Pérez de la Mora and Massieu, 1969).

From the early biochemical studies of Killam and his co-workers it seemed very likely that thiosemicarbazide-induced convulsions were a direct consequence of a decrease in cerebral GABA content subsequent to inhibition of GAD (Killam, 1957; 1958; Killam, Dasgupta and Killam, 1960; Killam and Bain, 1957). This simple explanation of the mechanism of hydrazide-induced convulsions was challenged in later experiments (Balzer, Holtz and Palm, 1960; Baxter and Roberts, 1960; Maynert and Kaji, 1962) in which it was shown that there was a lack of correlation between the time of maximum decrease in GABA content of the brain and the time of maximum seizure susceptibility. The situation has been clarified somewhat by a recent comprehensive study in which various hydrazides were administered in doses such that convulsions occurred after an equal latent period with all the drugs. When this was done there were several common biochemical findings with all the drugs; firstly that the rate of decrease of GABA concentration in the brain was similar for all the drugs; secondly, that GAD was always inhibited, though to varying degrees; and thirdly that there was very little inhibition of GABA-T (Wood and Abraham, 1970).

A similar spectrum of biochemical findings has been seen in rats which developed convulsions as a result of exposure to oxygen at high pressure (Wood and Watson, 1963, 1964; Wood, Watson and Stacey, 1966; Wood, Watson and Ducker, 1967). In these experiments the severity of the convulsions correlated well with the extent of the decrease in brain GABA content.

However the situation is further complicated by the existence of two drugs, amino-oxyacetic acid (AOAA) and l-glutamyl hydrazide, which raise tissue GABA levels at the same time as they cause convulsions (Tapia et al., 1969). At a convulsive dose level these drugs inhibit both GAD and GABA-T, but the dosage of AOAA can be adjusted so that it effectively inhibits only GABA-T. At this dose level AOAA can protect animals against thio-semicarbazide-induced convulsions (De Vanzo, Greig and Cronin, 1961; Roa, Tews and Stone, 1964; Schumann, Paquette, Heinzelman, Vallach, De Vanzo, and Greig, 1962) though not against high pressure oxygen-induced convulsions (Wood and Watson, 1965). Again, the maximum protection from convulsions occurs at a different time from the maximum increase of brain GABA content (Kuriyama, Roberts and Rubinstein, 1966). The relationship between brain GABA concentration and seizure susceptibility is therefore a matter of some complexity (Baxter, 1969) although a drop in the tissues levels of GABA nearly always results in convulsions. As Tapia et al. (1969) have pointed out, inhibition of GAD seems to be constant feature of drugs which interfere with pyridoxal

phosphate coenzymes and also cause convulsions. The parodox of convulsions occurring in the face of elevated GABA levels in the brain might be explained by the existence of two different functional "pools" of GABA. GABA-T is principally a mitochondrial enzyme and Kuriyama and Roberts (1969) have speculated that it may be located principally in post-synaptic areas and glia, while GAD may be concentrated in pre-synaptic nerve endings. Inhibition of GABA-T might then raise the tissue level of GABA but have very little effect on the pre-synaptic pool of GABA which may be available for release at inhibitory synapses.

There would seem therefore to be a considerable body of evidence to support a link between GABA metabolism and convulsions. Whether this is a causal relationship is not known. Certainly a causal link would be consistent with the hypothesis that GABA is an inhibitory transmitter in the central nervous system.

In this section of the thesis I shall report on the development and application of a new micro-technique which allows the quantitative analysis of GABA and associated amino acids in microgram amounts of brain tissue.

Animal Models of Epilepsy

The most widely studied animal models for epilepsy are the convulsive states produced either by electroshock or by convulsant drugs such as pentylenetetrazole. These models have proved very useful in the study of anticonvulsant drugs. However the transitory nature of the convulsive states, and the fact that they are

being induced by agents which affect the whole brain suggest they may not be good models for studying the mechanisms involved in human epilepsy.

It has been shown that certain types of insult to the brain can produce long-lasting localised abnormalities of brain function very similar to those found in cases of focal epilepsy in humans. Openchowski in 1883 was the first to demonstrate that freezing a small circumscribed area of the surface of the cerebral cortex resulted in an "epileptic" focus at the site where the tissue was frozen. The momentary freezing of the tissue may be achieved by placing a very cold rod of a good conducting material on the surface of the brain until ice crystals are seen to form on the cortical surface (Keith and Bickford, 1954). Alternatively the surface of the brain may be cooled with an ethyl chloride spray (Morrell and Florenz, 1958). The lesion produced by such techniques starts to discharge within 1-3 hours. The spike discharge continues at a steady rate for some 12 hours, after which time it becomes less frequent. Intermittent paroxysmal discharges may continue in the rabbit for up to 6 weeks, and in the cat for up to 3 months.

Application of alumina cream to the cortex (either topically or by injection into the grey matter) results in a different type of epileptogenic lesion (Kopeloff, Barrera and Kopeloff, 1942) in that the seizure activity produced by this technique does not develop for several weeks. Once established however, the focus may continue to discharge abnormally for a period of years.

Many other agents including penicillin (Walker, Johnson and Kallross, 1945), tungstic acid (Blum and Liban, 1960) antimony, bismuth, ~~nickel~~, cadmium and nickel (Chusio and Kopeloff, 1962) have been used to produce epileptogenic lesions in the brain.

In this present study epileptogenic lesions in the cortex of the rat have been induced by the implantation of a small quantity of cobalt into the cortical grey matter (Dow, Fernandez-Guardiola and Manni, 1962; Fischer Holubar and Malik, 1968; Dow, Park, Pryor and Townsend, 1971; Dow, McQueen and Townsend, 1972). With this lesion clinical seizure activity is evident within days, and evidence of spike discharges persists for a period of months. There is also evidence from electrocorticogram records that a secondary epileptic focus (the "mirror" focus first described by Morrel and Baker, 1961) is set up in the corresponding area of the contralateral cortex. In this section of my thesis I shall report the results of the analyses of GABA and associated amino acids in both primary and secondary epileptic foci.

At the outset of this work I was interested primarily in the relationship between GABA and epilepsy but the method which I chose to measure GABA levels in the brain showed itself to be readily applicable to the analysis of other amino acids as well as GABA. Glutamate, glutamine, glycine and aspartate were therefore also included in this study. Glutamate was included firstly on the ground that it is the immediate precursor of GABA (Fig.3:1) and secondly because it may have an excitatory transmitter

function in the central nervous system (reviews - Krnjevic, 1970; Johnson, 1972). Glutamine was of interest principally as a reservoir of glutamate (Fig. 3:1), while glycine (e.g. Werman and Aprison, 1968; Curtis and Johnson, 1970; Logan and Snyder, 1971; Iversen and Bloom, 1972) and aspartate (e.g. Curtis and Watkins, 1963; Logan and Snyder, 1971) have been proposed as candidates for, respectively, inhibitory and excitatory transmitter roles in the central nervous system.

METHODS

Choice of method to analyse GABA levels in a model epileptic focus

A cobalt implant in the rat cortex, such as I have used in this study (see pages 250 to 252), produces discrete histological changes in a small area of cortex surrounding the implant(1 mm radius 11 days after the implantation). Abnormal epileptiform activity arises both from the site of the cobalt implant (the primary focus) and from the secondary "mirror" focus in the contralateral cortex. It seemed possible therefore that biochemical changes might be restricted to these sites. It also seemed possible that only a relatively small number of cells in the focal area might in fact be "epileptic". If these "epileptic" cells were scattered over the focal area it might be very difficult to detect any significant biochemical changes because of "dilution" with normal cells. However if the "epileptic" cells were grouped together in a distinct zone within the focus it might be possible to detect significant biochemical changes by examining that zone in isolation. To allow examination of this latter possibility required that I use a method of assay which would allow the estimation of GABA in sub-milligram amounts of brain tissue.

Jakoby and Scott (1959) have reported a highly specific enzymic assay procedure for GABA, and this has been employed by Baxter and Roberts (1959, 1960) and Fahn and Côté (1968) in studies of GABA levels in different brain areas. The method, as

applied by Fahn and Côté, has a detection limit of about 1 nanomole of GABA and therefore might allow the estimation of GABA in about 1 mg of cortical tissue. Since I intended eventually to examine even smaller areas of tissue within the focal area this method was not considered suitable.

A more sensitive enzyme assay for GABA is that reported by Otsuka, Obata, Miyata and Tanaka (1971). This technique combines the method of Jakoby and Scott (1959) with the enzymic cycling technique of Lowry, Passoneau, Schulz and Rock (1961), and permits the measurement of as little as 2×10^{-14} moles of GABA. Using this method Otsuka et al. (1971) carried out GABA analyses on single isolated nerve cell bodies. However this method has the disadvantage of requiring five different highly purified enzymes and several highly purified substrates. It was therefore excluded on the grounds of high complexity and cost. Another factor in its exclusion was that cobalt in the primary focal area might interfere with the enzymes involved in the assay.

Use of either of these two methods for GABA estimation would also have required the parallel development of entirely separate techniques for the other amino acids in which we were interested, namely glutamate, aspartate, glutamine and glycine. The standard techniques for automatic amino acid analysis using an ion-exchange column separation followed by a colorimetric reaction with ninhydrin were too insensitive. For instance a recent study of amino acid levels in human brain samples used about 100 mg of

tissue for a satisfactory analysis (Perry, Berry, Hansen, Diamond and Mok, 1971).

In 1970 Neuhoff and Weise reported a method for the preparation, separation and detection of dansyl derivatives of amino acids in brain tissue. This method was said to have a potential sensitivity for the estimation of amino acids in the picomole range, although as applied by Neuhoff and Weise it was only semi-quantitative.

Weber (1952) had first demonstrated that dansyl chloride could produce a stable, fluorescent, covalently-bonded conjugate with protein. Hartley and Massey (1956) attempted to use dansyl chloride as a fluorescent label for the amino acid group at the active centre of the enzyme chymotrypsin. In 1963 Grey and Hartley reported on the use of dansyl chloride as a reagent for end-group analysis of proteins and peptides. It was found to be 100 times more sensitive than the original fluorodinitrobenzene reagent introduced by Sanger (1949). The usefulness of dansyl chloride as a reagent for end-group analysis depends on three factors: the high yield of dansylated products; the stability of the dansyl-amino acid bond during acid hydrolysis of the protein; and the high quantum yield of dansyl-amino acid fluorescence within the visible range of the spectrum. Since Grey and Hartley's original report the technique has become a standard method of end-group amino acid analysis (review; Hartley, 1970).

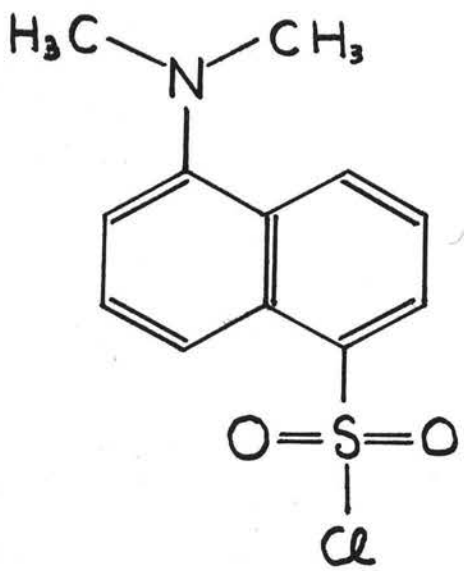
As the dansylation method appeared to be potentially suitable for estimating GABA and other amino acids with the required

sensitivity it was studied further.

Dansyl Chloride

Dansyl chloride (Fig. 3:2), or to give it its full title 1-dimethylaminonapthalene-5-sulphonyl chloride, behaves as a typical aromatic sulphonyl chloride, reacting with a wide variety of bases such as primary or secondary amines, phenolic hydroxyls, thiols and imidazoles. Tertiary amines do not react with dansyl chloride but catalyse the reaction with primary and secondary amines. The bonds formed between dansyl chloride and thiol and imidazole groups are unstable. The reaction between the primary amino group of an amino acid and dansyl chloride is highly dependent on the pKa of the amino acid and the pH of the solution in which the reaction takes place. When the pH is low and the amino group protonated, dansyl chloride preferentially hydrolyses water. As the pH rises however, hydroxyl ions become strongly competing nucleophiles. Hartley (1970) has prepared a graph showing theoretical yields of different amino acids with dansyl chloride at different pH. The optimal range for dansylation of most amino acids lies between pH 9 and 10. Grey and Hartley (1963) found that carrying out the dansylation of end-group amino acids in 50% aqueous acetone produced solubilisation of the dansyl reagent and suppressed the ionisation of the amino groups on the amino acids.

The important reactions in respect of the dansylation of tissue amino acids are shown in Fig. 3:3. The first reaction



1-DIMETHYL AMINO NAPTHALENE-5-SULPHONYL CHLORIDE
(DANSYL CHLORIDE -- $ArSO_2Cl$)

FIG. 3:2 The structure of dansyl chloride.

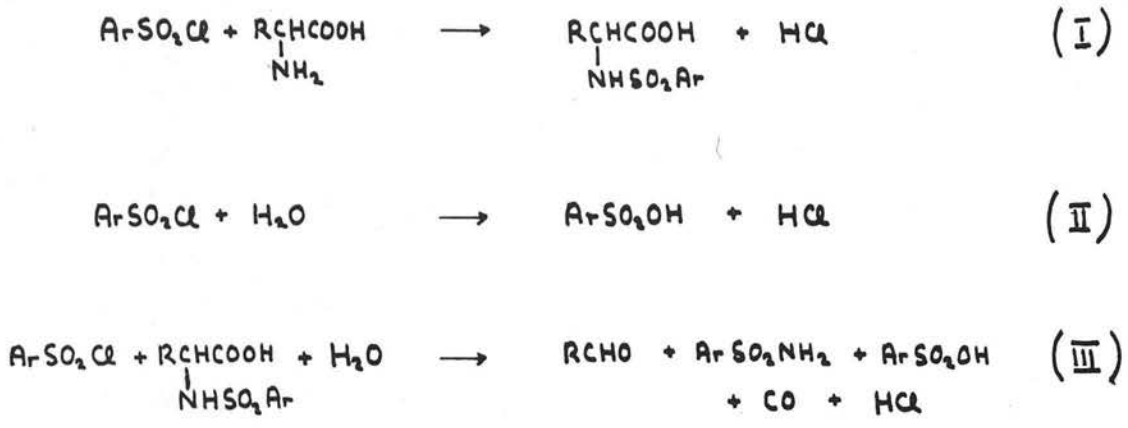


FIG. 3:3 Reactions of dansyl chloride

The three reactions illustrated occur in buffered (pH 8.5 - 10) aqueous acetone (50%) solution between:-

I Dansyl chloride and an amino acid
II Dansyl chloride and water
III Dansyl chloride and a dansyl-amino acid.

is that between dansyl chloride and the primary amino group of an amino acid. At a pH between 9 and 10 the dansyl chloride attacks the amino group of the amino acid forming a dansyl amino acid and hydrochloric acid. In the case of an aromatic amino acid any phenolic hydroxyl groups or imidazole groups may also be dansylated.

The second reaction shows the hydrolysis of dansyl chloride by ~~hydrolysing~~ water with the formation of dansyl hydroxide (1-dimethylaminonapthalene-5-sulphonic acid) and hydrochloric acid.

The kinetics of these first two competing reactions have been studied by Spivak, Shcherbukhin, Orlov and Varshavsky (1971).

The third reaction between dansyl chloride and dansyl amino acids was first reported by Neadle and Pollit (1965). In this reaction a dansyl amino acid derivative is degraded with the formation of: an aldehyde derived from the amino acid; dansyl amine from the dansyl moiety; and carbon monoxide from the carboxyl group of the amino acid.

This last reaction introduces a complicating factor in the establishment of conditions which will give the optimum yield of dansyl amino acid since both reactions I and III are favoured by an excess of dansyl chloride (Spivak et al., 1971).

Equipment used in the extraction, dansylation and quantitative estimation of amino acids in brain tissue.

Stage I - Homogenisation, protein precipitation and dansylation.

1. Conical polypropylene reaction tubes, capacity 1.5 ml (Eppendorff).
2. Glass homogenisation pestle, ground at one end to fit into the above tubes.
3. High speed centrifuge, capable of producing 20,000 g (Mistral, M.S.E., fitted with high speed head).
4. 2, 5 and 10 µl microcapillaries (Drummond).
5. Glass micro-reaction tubes, capacity approximately 100 µl, length approximately 20 mm (prepared from glass capillary tubing, internal diameter 2.5 mm, external diameter 3.5 mm).
6. Rubber caps for micro-reaction tubes (the rubber washers from the plunger heads of 1 ml polyethylene syringes (Becton, Dickinson and Co. Ltd)).
7. Heating block, 37°C (Eppendorff).
8. Source of nitrogen gas.
9. Calcium chloride drying tower.

Stage II - Chromatography

10. Polyamide micro-chromatography plates (3.5 x 3.5 cm) prepared from 15 x 15 cm polyamide plates (a layer of poly-E-caprolactam applied to both sides of a solvent-

resistant polyester base). The plates obtainable from Carl Schleicher and Schull were found to give slightly better resolution of the dansyl derivatives than those obtainable from B.D.H.

11. Fine glass micro-capillary with flamed tip (tip diameter 0.05 - 0.15 mm), prepared from standard melting-point tubes.
12. Stereo microscope (Zeiss).
13. Micro-chromatography tanks (Quickfit glass weighing bottles, 4.7 cm diameter, 5 cm depth).

Stage III - Identification and assay of dansyl amino acid spots.

14. Source of short wavelength uv. light (254 nm).
15. Liquid scintillation counter (Nuclear Chicago, Mark II).

Materials used in the development and application of the dansylation method for the quantitative estimation of amino acids in brain tissue.

Radiochemicals from the Radiochemical Centre - Amersham

	Specific Activity	Activity as despatched
^{3}H - dansyl chloride	5600 ci/mole	250 µci in 500 µl benzene
^{14}C - glutamic acid	260 "	50 µci in 800 µl 2% ethanol solution
^{14}C - aspartic acid	227 "	50 µci in 1000 µl "
^{14}C - glutamine	45 "	50 µci in 800 µl "
^{14}C - GABA	204 "	50 µci in 500 µl "
^{14}C - glycine	108 "	50 µci in 1000 µl "
^{14}C - hydroxyproline	38 "	10 µci (solid)

Chemicals

Dansyl chloride, dansyl glutamic acid, dansyl glutamine, dansyl glycine, and dansyl GABA were obtained from B.D.H.

dl-α-alanine, dl-2-amino-n-butyric acid, l-arginine monohydrochloride, dl-aspartic acid, l-cysteine hydrochloride, l-cystine, dl-3,4-dihydroxyphenylalanine, l-glutamic acid, glycine, l-histidine monohydrochloride, l-hydroxyproline, l-leucine, dl-iso-leucine, dl-nor-leucine, l-lysine monohydrochloride, dl-methionine, dl-ornithine monohydrochloride, dl-β-phenylalanine, l-proline, dl-serine, dl-threonine, dl-tryptophan, dl-tyrosine, dl-valine, and d-amino-n-butyric acid (GABA) were all obtained from B.D.H.

2-aminoethanol (ethanolamine), l-homocarnosine sulphate, dl-norvaline, glutathione (reduced form), taurine, and n-acetyl-dl-aspartic acid were obtained from Sigma Chem. Co. Ltd.

Solutions

0.48 N perchloric acid

1.5 M potassium carbonate solution

0.2 M potassium bicarbonate solution

24×10^{-3} M dansyl chloride solution in redistilled acetone (only used in preliminary experiments)

5.69×10^{-3} M dansyl chloride solution in redistilled acetone.

^{3}H - dansyl chloride in benzene (250 μci/ml).

Stock solution of ^{14}C - amino acids

The 10 µci of ^{14}C - hydroxyproline (solid) was taken up in 200 µl water.

Using this solution of ^{14}C - hydroxyproline and the solutions of the other ^{14}C - amino acids as delivered from the Radiochemical Centre the following stock solution was prepared.

			Activity of each amino acid in 2 µl of this solution
C^{14} - Glutamate	solution	20 volumes	31.2 nci
C^{14} - Aspartate	"	20 "	25.0 "
C^{14} - Glutamine	"	15 "	23.4 "
C^{14} - Glycine	"	10 "	12.5 "
C^{14} - Hydroxyproline	"	10 "	12.5 "
C^{14} - GABA	"	5 "	12.5 "

Scintillant Solution

1,4-di- 2 -[(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP) and 2,5-diphenyloxazolyl (PPO) were obtained from Koch-Light Co. Ltd.

Scintillant solution was prepared by adding 0.112 g dimethyl POPOP and 4.25 g PPO to 1 litre of toluene.

Solvent Systems

1. formic acid : water (1.5 : 98.5) by volume
2. benzene : glacial acetic acid (9 : 1).

Development of techniques for dansylation and quantitative estimation of amino acids in sub-milligram amounts of brain tissue.

The separation of dansyl amino acids

In the early work on the end group analysis of proteins the dansylated amino acids were separated by paper electrophoresis (Grey and Hartley, 1963). Subsequently thin-layer techniques were introduced and Deyl and Rosmus (1965) studied the separation of dansylated amino acids in several different solvent systems using either silica gel or alumina as a carrier. In 1967 Woods and Wang reported excellent separation of dansylated amino acids on polyamide chromatography plates. Micro-plates of this material measuring 3 x 3 cm have been employed by Neuhoff and Weise (1970) and Bruton and Hartley (1970). In both these studies the plate was first run in a 1.5% (v:v) aqueous solution of formic acid. The plate was then carefully dried and re-run at 90° to the original direction in a mixture of benzene and glacial acetic acid (9:1, v:v). Crowshaw, Jessup and Ramwell (1967) and Hartley (1970) have reported the use of a third solvent system of ethyl acetate : methanol : glacial acetic acid (20:1:1, v:v:v) to achieve better separation of some poorly defined pairs of dansylated amino acids.

Neuhoff and Weise (1970) stated that as little as 10^{-12} moles of dansylated amino acids could be separated on polyamide micro-chromatography plates. This was the method used in this present study.

Polyamide chromatography plates (15 x 15 cm) were obtained from B.D.H. These plates consist of a layer (50 microns thick) of poly-ε-caprolactam applied to both sides of a solvent resistant polyester base. Neuhoff and Weise (1970) used these plates cut into 3 x 3 cm squares but I found that a size of 3.5 x 3.5 cm squares was more easily handled during chromatography.

Quickfit glass weighing bottles (see page 212) were found to make very satisfactory micro-chromatography tanks for these 3.5 x 3.5 cm micro-chromatography plates.

Standards of dansyl-glutamic acid, dansyl-glutamine, dansyl-GABA and dansyl-glycine were made up in 0.2 M potassium bicarbonate solution to give 10^{-4}M solutions of each of these four dansylated amino acids. The chromatographic separation of these dansyl amino acids was then investigated.

Application of very small volumes (0.1 - 0.3 microlitres) of these standard solutions to the polyamide micro-plates was a major problem. Because of the small total area of the plate the spot size had to be kept below a diameter of 0.5 mm. Also one had to avoid physical damage to the surface of the plate during the application procedure. The procedure eventually used was application of the solution from a fine glass capillary. For satisfactory working the tip diameter had to be within the range of 0.08 - 0.12 mm. To prevent damage to the plate surface the tip of the capillary was slightly flamed. By manual manipulation in the visual field of a stereo-microscope the capillary (filled

by capillary attraction) was lightly touched onto the surface of the plate causing a very small volume of fluid (less than 0.01 µl) to run out of the capillary. It was found helpful to have a blower (operated by a variable foot switch) blowing a stream of cold air over the working area.

Homogenisation and extraction of free amino acids from tissue

Neuhoff and Weise (1970) homogenised 20 mg of brain tissue in 0.05 M sodium bicarbonate solution and then precipitated protein with trichloroacetic acid (TCA). This agent cannot be readily removed from the supernatant after the precipitation of protein. Since the dansylation of the amino acids requires an alkaline pH (between pH 9-10) the TCA must first be neutralised with alkali. As a result the final dansylated TCA extract contains a considerable amount of TCA salts. When this dansylated extract is applied to the plate the spot rapidly becomes "loaded" with these TCA salts. These salts cause streaking of the chromatogram.

It was decided that this problem made TCA an unsuitable protein precipitating agent. Three protein precipitating agents which can readily be removed from the supernatant extract are ethanol, acetone and perchloric acid. Of the three, perchloric acid gives the most efficient protein precipitation. Saifer (1971) has recommended perchloric acid extraction as the best method overall for the quantitative estimation of free amino acids in brain tissue.

It was therefore decided to homogenise the tissue in perchloric acid and subsequently neutralise the solution with a strong solution of potassium carbonate, thus precipitating perchlorate as its highly insoluble potassium salt. It was found that this technique resulted in a lower concentration of inorganic salts in the final dansylated extract. This allowed the application of a greater volume of dansylated extract to the plate without causing streaking of the chromatogram.

Conditions of Dansylation

Gray and Hartley (1963) reported that a 50% solution of acetone in bicarbonate buffer (pH 9) was a suitable medium for the dansylation of end-groups in proteins. The presence of the acetone achieved stabilisation of the dansyl chloride and inhibited the protonation of the amino acid. Spivak et al. (1971) studied the dansylation of glycine, serine, proline and leucine. Using 50% acetone bicarbonate buffer they found that a 100-500 fold excess of dansyl chloride over amino acid resulted in optimal yields. For the quantitative estimation of amino acid mixtures they recommended adding one volume of 30×10^{-3}M dansyl chloride solution in acetone to one volume of the buffered mixture of amino acids (amino acids 10^{-3}M or below). The occurrence of reaction III (Fig. 3:3), namely the degradation of a dansyl amino acid by dansyl chloride, could be minimised by carrying out the dansylation at room temperature and limiting the reaction time to 40 minutes.

It was decided to use these conditions in the present study. However it was found that dansyl chloride solutions (in acetone) of above 24 x 10^{-3}M resulted in precipitation of dansyl chloride when they were added to equal volumes of aqueous buffer solutions. A 24 x 10^{-3}M solution of dansyl chloride in acetone was therefore used in preliminary investigations.

Preliminary separation and concentration of dansyl-amino acids

The possibility of extracting dansyl-amino acids from the final acetone/buffer mixture has been investigated. It was found that the carboxyl groups on the dansyl-amino acids prevented their extraction into organic solvents. However it was noted that dansyl chloride (strong yellow coloration) was extracted into di-ethyl ether.

A mixture of amino acids was dansylated and the final dansylated mixture was extracted with 2 volumes of di-ethyl ether, giving a yellow coloured ether extract and a colourless aqueous residue. An aliquot of the ether extract was spotted onto a polyamide micro-plate which was then developed as described on page 222 . Under short wavelength u.v. light the developed chromatogram showed only two discrete spots. One of the spots was identified as dansyl hydroxide by its strong blue fluorescence. The other, a very much fainter yellow-green spot, was identified as dansylamine. The extracted dansyl chloride produced a strong diffuse fluorescence principally at the head of the second solvent front directly above the origin.

Following the ether extraction the residue containing the dansyl-amino acids was taken to dryness under dry nitrogen at 37°C. The dansyl-amino acids were then extracted with ethanol. Ethanol was chosen to extract the dansyl-amino acids for three reasons: firstly because these compounds are soluble in ethanol; secondly because inorganic salts are relatively insoluble in ethanol and therefore much of the buffer salt was left behind as a solid residue; and thirdly because the application of an ethanol solution to the polyamide chromatography plates was more easily controlled than was the application of an aqueous acetone solution. When the ethanol extract was spotted onto a polyamide plate and chromatographed as described on page 222 examination of the developed plate under u.v. light showed that the ether wash and the ethanol extraction had resulted in several improvements. In the first place the ether wash removed much of the dansyl-hydroxide and dansyl-amine, neither of which I was interested in. In the second place the ethanol extraction allowed more of the dansyl amino acids to be spotted onto the plate without the danger of applying too much salt. As a result the developed chromatogram showed more intense and discrete spots with an absence of streaking (Fig. 3: 5). Another important action of the ether wash was to remove excess reagent (dansyl chloride) thus preventing continuing degradation of dansyl-amino acid by attack with dansyl chloride (reaction III, Fig. 3: 3).

Preparation of map of separated dansyl amino acids

Before progressing to the analysis of amino acids in tissue it was necessary to prepare a map showing the relative positions of the dansyl derivatives of the amino acids after chromatography on a polyamide plate.

The principal amino acids likely to be encountered in brain tissue, along with a few unnatural synthetic amino acids, (see check list for Fig. 3:4) were made up individually in 0.2 M potassium bicarbonate buffer and then aliquots of the individual solutions were mixed so that the final mixture was 2×10^{-4}M with respect to each individual amino acid.

For locating the position of an individual amino acid derivative, a 1 ml aliquot of the above mixture was placed in a 15 ml glass centrifuge tube. A small volume of the stock solution of the amino acid under study was then added to the 1 ml aliquot of the mixture to give a ten-fold excess of that particular amino acid over the others in the mixture (i.e. 2×10^{-3}M as opposed to 2×10^{-4}M). The mixture was then dansylated with an equal volume of dansyl chloride solution in acetone (24×10^{-3}M), for 40 minutes at room temperature in the dark. After this period excess dansyl chloride reagent was removed by washing with 2 volumes of di-ethyl ether. The aqueous residue was then evaporated under dry nitrogen at 37°C and extracted with 1 ml of ethanol. 0.1 µl of the ethanol extract was then spotted onto the corner of a polyamide plate. The plate was first run in

ascending fashion in 1.5% formic acid solution (solvent I). It was then carefully dried and re-run at 90° to the original direction in benzene : glacial acetic acid (9:1, v:v) (solvent II). After drying, the plate was examined under u.v. light. The spot pertaining to the amino acid under study stood out from all the others by its more intense fluorescence.

This procedure was repeated with all the amino acids and from these studies the map shown in Fig. 3:4 was prepared. It was apparent from these early studies that modifications would have to be made to the conditions of the chromatography in order to separate firstly dansyl glutamine from dansyl serine and dansyl threonine; and secondly dansyl aspartate from dansyl glutamate. It was not considered necessary to attempt to separate dansyl GABA from dansyl α-amino-n-butyric acid since only traces of this latter compound can be detected in the brain (Perry, Hansen, Berry, Mok and Lesk, 1971).

Several different solvent systems were tried in an attempt to improve the separation of dansyl-glutamine, dansyl-aspartate and dansyl-glutamate. The solvent systems are shown below:-

(1) water : formic acid (98.5 : 1.5)
(2) benzene : glacial acetic acid (9 : 1)
(3) ethyl acetate : methanol : glacial acetic acid (20 : 1 : 1)
(4) n-heptane : n-butanol : glacial acetic acid (3 : 3 : 1)
(5) n-butanol : pyridine : glacial acetic acid : water (30 : 20 : 6 : 24)
(6) benzene : pyridine : glacial acetic acid (16 : 4 : 1)

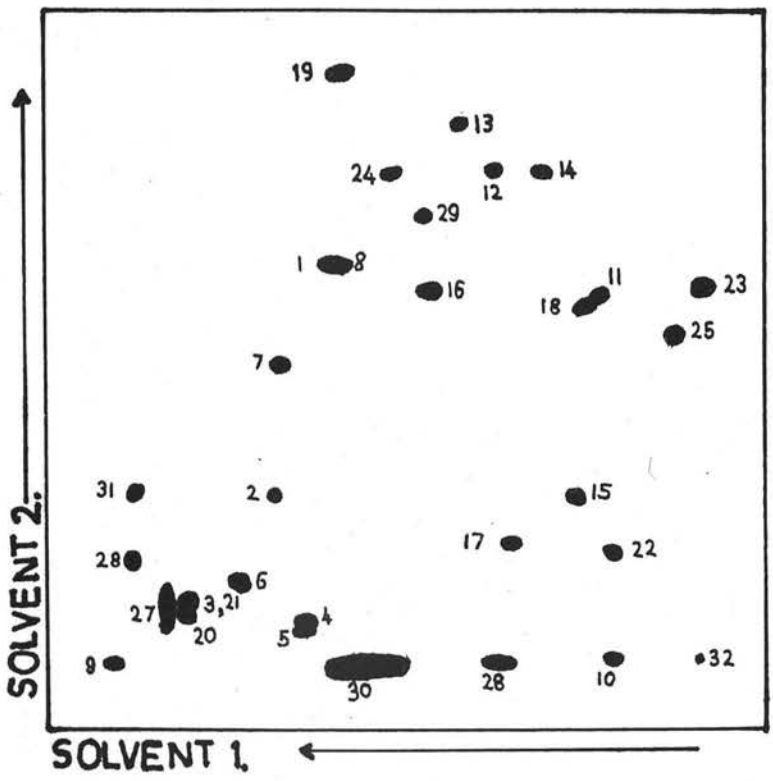


FIG. 3:4 Dansylated Amino Acid Map

Solvent 1 : 1.5% Formic Acid

Solvent 2 : Benzene : Glacial Acetic Acid. (9:1)

1. γ -Amino Butyric Acid
2. Glycine
3. L-Glutamine
4. L-Glutamic Acid
5. DL-Aspartic Acid
6. L-Hydroxy Proline
7. DL-Alanine
8. DL-α-Amino Butyric Acid
9. L-Arginine
10. L-Cysteine
11. L-Histidine
12. L-Leucine
13. DL-Isoleucine
14. DL-Norleucine
15. L-Lysine
16. DL-Methionine
17. DL-Ornithine
18. DL- β- Phenyl Alanine
19. L-Proline
20. DL-Serine
21. DL-Threonine
22. DL-Tryptophan
23. L-Tyrosine
24. DL-Valine
25. L-Homocarnosine
26. Taurine
27. Glutathione
28. Aminoethanol
29. DL-Norvaline
30. Dansyl-Hydroxide
31. Unknown
32. Starting Point

(7) chloroform : benzyl alcohol : glacial acetic acid (70 : 30 : 3)

(8) chloroform : ethanol : glacial acetic acid (38 : 4 : 3)

Solvent systems (5) to (8) gave poor separation and definition of the dansyl-amino acid spots and were not investigated further. Solvent system (3) which has been reported by Hartley (1970) to separate dansyl-glutamate from dansyl-aspartate was no more successful in this respect than solvent system (2). Solvent system (4) was more successful in separating dansyl-glutamate from dansyl-aspartate but it carried both dansyl-GABA and dansyl-glycine almost at the solvent front where they became mixed with other dansyl derivatives. It was found that developing the chromatogram in solvent system (2) with the lid of the tank off for 9 minutes resulted in separation of dansyl-glutamate from dansyl-aspartate. Under these conditions the solvents evaporated about half-way up the plate and as a result the dansyl derivatives with a low Rf value continued to advance up the plate while the derivatives with a high Rf value remained half-way up the plate at the solvent front. The lid of the tank was replaced after 9 minutes and the solvent front allowed to reach the top of the plate. Fig. 3:5 shows a mixture of dansylated amino acids which have been chromatographed in this way following an initial separation in solvent system (1). The extended run in solvent system (2) separated dansyl-glutamate from dansyl-aspartate, and dansyl-serine from dansyl-glutamine and dansyl-threonine.

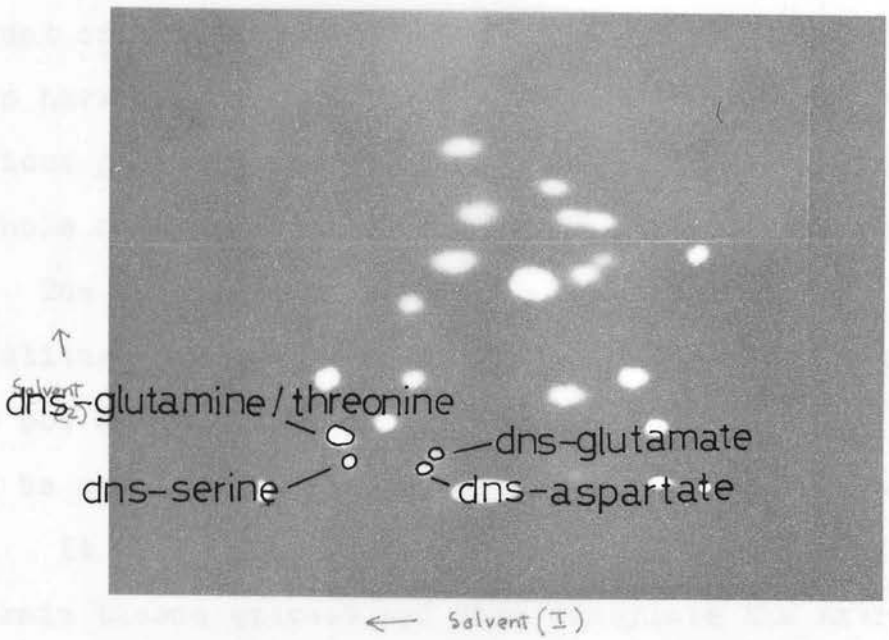


FIG. 3:5 : Chromatogram of a mixture of dansyl-amino acids after a normal development in Solvent (I) and an extended* development in solvent system (2). Shows separation of

(a) dansyl-glutamate from dansyl-aspartate
(b) dansyl-glutamine/threonine from dansyl-serine.

*See text page 222 for explanation.

Recovery of ^{14}C-amino acids through the method

Neuhoff and Weise (1970) had shown that the amount of dansylated amino acid on a plate could be quantified by using a radioactively-labelled dansyl chloride of known specific activity and counting the activity of the dansylated amino acid spot. However before such information could be applied to estimating the amount of amino acid originally present in a sample one would also have to estimate the recovery of amino acid through the various procedures of dansylation and chromatography. Two methods of accomplishing this have been investigated.

The amino acid hydroxyproline is found principally as a constituent of collagen and gelatin; its occurrence in brain has not been reported. The dansyl derivative of hydroxyproline can be readily separated from other dansyl-amino acids (Figs. 3:4, 5). It was intended to add a known amount of hydroxyproline to a brain tissue extract and then dansylate the extract with radioactively-labelled dansyl chloride. Following chromatography of the dansylated extract the recovery of hydroxyproline through the method could be calculated from the activity of the dansyl-hydroxyproline spot. However before the recovery figure for hydroxyproline could be applied to the other amino acids, it would have to be shown that the recoveries of the other amino acids varied in constant proportion to the recovery of hydroxyproline.

This question, along with other basic questions as to the effect of various parameters on the yield of the ^{14}C-dansylation

reaction, was investigated, using ^{14}C-amino acids. ^{14}C-glutamic acid, ^{14}C-glutamine, ^{14}C-aspartic acid, ^{14}C-glycine, ^{14}C-GABA, and ^{14}C-hydroxyproline were obtained from the Radiochemical Centre, Amersham.

An aliquot (5 or 10 µl) of a mixture (see below for composition of mixture) of these ^{14}C-amino acids in 0.2 M potassium bicarbonate buffer was carefully placed inside a small glass micro-reaction tube (see list of apparatus, no.5, page 211). An equal volume of a 24 x 10^{-3}M solution of dansyl chloride in acetone was added to the tube which was then thoroughly agitated, capped, and left in the dark at room temperature for 40 minutes. After this time the dansylated mixture was washed with two volumes of diethyl ether, taken to dryness under dry nitrogen at 37°C, and extracted with 10 µl of ethanol. Using a 1 µl Drummond microcapillary (calibrated by length on the basis of its having a uniform internal diameter) a known volume of ethanol extract (between 0.1 and 0.5 µl) was transferred to a fine glass microcapillary for application to a polyamide chromatography plate. After chromatography the various dansylated amino acid spots were identified under u.v. light and ringed with a soft pencil. The ringed spots were then cut from the plate, using small sharp scissors, and placed in standard 20 ml glass scintillation vials. 1 ml of ethanol was added to each of the vials which were then violently agitated (Whirlimix). 10 ml of a toluene-based scintillant solution (see page 214) were added to the vials which were then

counted in a liquid scintillation counter (Nuclear Chicago, Mark II) at ^{14}C settings. The efficiency of counting was determined by a channels-ratio method (Baillie 1960).

If, after counting, the small portions of polyamide plate were carefully removed from the vials and the vials then re-counted it was found that there was no detectable drop in the counting rates. This demonstrated that the ^{14}C-dansyl-amino acids had been eluted from the polyamide layer by the ethanol and were homogeneously distributed throughout the scintillant solution. Toluene scintillant solution alone did not elute the dansyl-amino acids from the polyamide layer.

The composition of the ^{14}C-amino acid mixtures used in these recovery experiments was such that after dansylation and chromatography, the spot pertaining to each individual amino acid was both visible under u.v. light and sufficiently radioactive to be readily counted in a 20 minute counting time (i.e. greater than 10 times the background level of activity).

Table 3:1 shows the results of an experiment in which the amount of ^{14}C-labelled dansyl-amino acid actually recovered has been compared with the theoretical 100% recovery assuming complete dansylation of amino acid and no manipulative losses of ^{14}C-label throughout the method. Glutamate and aspartate have been shown together because the fluorescence of their spots on this occasion was so weak that it was not possible to tell clearly where one stopped and the other started. The joint recovery of both (5.7%) was very poor compared with that of the other amino acids (> 25%). This finding was repeated in other similar experiments.

TABLE 3:1 : Dansylation experiment with ^{14}C-amino acids demonstrating low recovery of aspartate and glutamate.

	D.p.m. expected[1]	D.p.m. obtained	% Recovery
Hydroxyproline	7840	5670	72.5
GABA	15680	8927	57.0
Glutamine	19600	6881	35.1
Glycine	15680	4260	27.2
Glutamate } Aspartate }	105800	5994	5.7

[1] The expected d.p.m. have been calculated on the assumption of complete dansylation and no manipulative losses through the method.

The other important finding of these early recovery experiments was that the recovery of one particular amino acid could vary independently of the others. This may have been due to slight differences in the pH at which the dansylation was carried out, since among other factors governing the degree of dansylation are the pKa of the amino acid and the pH of the reaction mixture. Whatever the reason, these experiments demonstrated that hydroxyproline could not be used as an internal standard to give a general figure for the recovery of amino acids through the dansylation procedure.

In attempts to improve the recoveries of glutamate and aspartate it was found that a reduction of the time of dansylation to 30 minutes almost doubled the recoveries of these two amino acids to 10%. This suggested that some of the dansyl-glutamate and dansyl-aspartate formed was subsequently being degraded (reaction III Fig. 3:3).

Another step in the method which was investigated was the ethanol extraction of dansyl glutamate and dansyl aspartate from the evaporated dansylation mixture. The residue remaining after an ethanol extraction was completely taken up in 50% acetone and water. The ethanol extract and the aqueous acetone solution were spotted onto separate polyamide plates. These were then developed in solvent systems (1) and (2) as before.

By counting the activity of the dansylated amino acid spots it was found that ethanol satisfactorily extracted dansyl-GABA,

dansyl-glycine, dansyl-glutamine and dansyl-hydroxyproline but failed to extract more than 30% of the dansyl-glutamate and dansyl-aspartate.

Because of this shortcoming in the ethanol extraction technique I considered simply spotting up the final aqueous acetone mixture but this had the disadvantage of being difficult to apply. It also resulted in a heavily salt-laden chromatography plate. It was decided to try the acetone : glacial acetic acid (3:2, v:v) extraction which had been used by Woods and Wang (1967). This solution was found to leave some of the salts undissolved and was very readily applied to the chromatography plates. It was also decided to reduce the dansyl chloride concentration to 5.7×10^{-3}M (Briel, Neuhoff and Maier, 1972). These two steps resulted in a much improved recovery of glutamate and aspartate (Table 3:2).

Recovery of ^{14}C-amino acids from a homogenate of rat cortical tissue

Having established satisfactory dansylation techniques for pure solutions of amino acids I then investigated the dansylation of ^{14}C-amino acids added to samples of brain tissue (rat cortex). Much of the methodology for these experiments was similar to that currently used in the analysis of tissue amino acids (full description pages 239 to 248). Therefore at this stage I shall only outline the approach to these experiments.

A small piece of rat cortical tissue, approximately 1 mg in weight, was placed on the inside wall of a glass micro-reaction

TABLE 3:2 : Dansylation experiment with ^{14}C-amino acids demonstrating satisfactory recovery of six amino acids.

	D.p.m. expected[1]	D.p.m. obtained	% recovery
Hydroxyproline	587	405	69.0
GABA	1175	689	58.6
Glycine	1175	683	58.1
Glutamine	1470	565	38.5
Aspartate	3525	1257	35.7
Glutamate	4410	1442	32.7

Footnotes : (1) See Footnote (1) Table 3:1 (page 229)

(2) The levels of activity in this table are much lower than in Table 3:1. This merely reflects the use of lower levels of activity in the light of experience gained from earlier experiments.

tube. It was then homogenised in 15 µl of 0.48 N perchloric acid using a small rod of perspex (1.8 mm diameter) shaped to fit the micro-reaction tubes. A 5 µl aliquot of a ^{14}C-amino acid mixture was then added to the tube which was left at 4°C for 20 minutes to complete the process of protein denaturation. The tube was then centrifuged at 20,000 g and a 10 µl aliquot of the protein-free supernatant was removed to another micro-reaction tube and neutralised with 2 µl 1.5 M potassium carbonate solution. The insoluble potassium perchlorate was spun down and a 10 µl aliquot of the supernatant was placed in another micro-reaction tube. An equal volume of dansyl chloride solution in acetone (5.7×10^{-3}M) was added to this tube which was then left for 30 minutes at room temperature in the dark. The contents of the tube were then taken to dryness under dry nitrogen at 37°C and extracted into 10 µl acetone : glacial acetic acid solution (3:2, v:v). A known volume (0.1 - 0.5 µl) of this extract was then spotted onto a polyamide plate and chromatographed as before. The plate was then examined under u.v. light. An example of a "tissue" plate is shown in Fig. 3:6. As the photograph shows, certain of these dansylated amino acid spots are very intense compared with others, reflecting the fact that certain amino acids are present in tissue in a free form at much higher concentrations. In this study it has not been possible to spend time in identifying all the dansyl derivatives present on a "tissue" plate. Only the dansyl-amino acid spots in which I was interested have been positively identified as in Fig. 3:6.

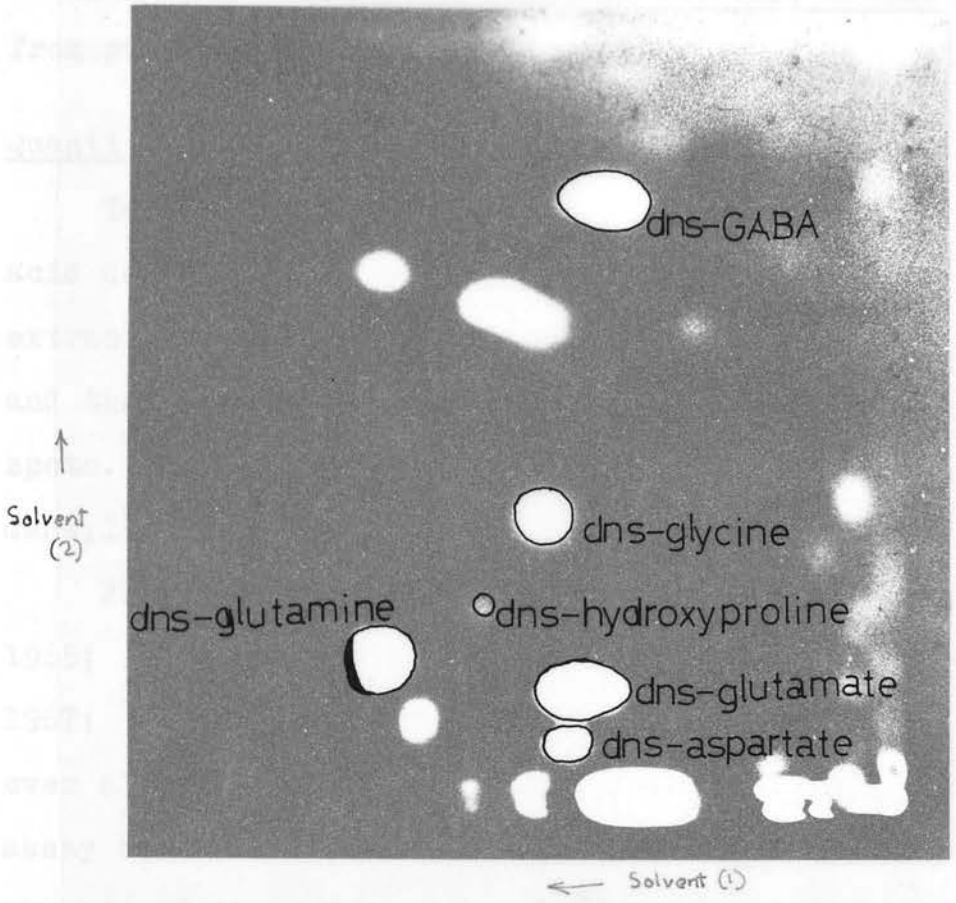


FIG. 3:6 : Chromatogram of dansylated-extract of tissue from rat cortex (control).

Solvent (I) normal development
Solvent (II) extended* development.

* See text page 22 for explanation.

The spots pertaining to the amino acids under study were marked and removed for counting of ^{14}C-activity. It was found that the recoveries of the ^{14}C-amino acids from the tissue homogenate were in the same range (30-80%) as the recoveries from pure solutions.

Quantitative assay of amino acid levels in tissue

Two methods have been used to assay separated dansyl-amino acid derivatives. Neuhoff and Weise (1970) dansylated tissue extracts with ^{14}C-dansyl chloride of known specific activity and then counted the activity of the separated dansyl amino acid spots. By this means they were able to calculate the amount of dansyl amino acid in the spot.

Fluorescence assay has also been used (Needle and Pollit, 1965; Seiler and Weichmann, 1966; Crowshaw, Jessup and Ramwell, 1967; Spivak, Shcherbukhin, Orlov and Varshavsky, 1971). However elution of a dansyl-amino spot and subsequent fluorescence assay of the dansyl-amino acid in solution is only possible with amounts of the order of 1×10^{-9} moles or higher (Crowshaw et al., 1967). Spivak et al. (1971) have reported a method for the assay of the fluorescent dansyl-amino acid "in situ" on a silica-gel chromatography plate. This method, as described, has a sensitivity of 1×10^{-11} moles. However it is based on the assumption that the dansyl derivatives are symmetrically distributed in their spots. Also, since the fluorescence of the dansyl-amino acids is strongly quenched by water, it requires very strict

control of humidity. Because of these exacting constraints this method was not considered to be suitable as a routine micro-method.

It was decided to combine the radioactive-labelling technique of Neuhoff and Weise (1970) with a technique for estimating the recovery of amino acid through the dansylation procedure. Use of hydroxyproline as an internal standard proved to be unsatisfactory. It was therefore decided to use added ^{14}C-amino acids as internal standards and carry out the dansylation with ^{3}H-dansyl chloride. Counting the spot of separated dansyl-amino acid for both ^{14}C and ^{3}H activity would then provide the following information : firstly, how much dansylated amino acid was present in the spot (from the ^{3}H activity); secondly, how much of the exogenously added ^{14}C-amino acid had been recovered (from the ^{14}C activity); and finally, how much of the amino acid was derived from the tissue. The ^{14}C activity could then be used to estimate the recovery of amino acid through the method, and this figure could then be applied to the amino acid derived from the tissue. The theory of the method (isotope dilution assay) and the actual mathematical analysis are described below. The practical aspects of the method as it is currently being used will be described later (pages 239 to 248).

Theory of method and mathematical treatment of results

The principle of the method, which forms one of the general class of isotope dilution assays, can be represented in four stages.

(1) Addition of known amount of ^{14}C-labelled amino acid, of known specific activity, to the "pool" of endogenous amino acid.

(2) Subsequent isolation of combined pool of endogenous and exogenous (^{14}C-labelled) amino acid.

(3) Measurement of specific activity of combined pool.

(4) Calculation of dilution of ^{14}C-labelled amino acid by non-radioactive endogenous amino acid, leading to calculation of the amount of endogenous amino acid originally present.

The method involves the basic assumption that there are no significant isotope effects at any of the various steps such as dansylation or chromatography. The specific activity (w.r.t. ^{14}C) of the combined pool of exogenous and endogenous amino acid therefore remains unaltered throughout the method.

Symbol Definitions

AAext = moles of exogenous ^{14}C-labelled amino acid added to each aliquot of perchlorate extract of tissue.

SAaa = specific activity of exogenously added ^{14}C-labelled amino acid (curies ^{14}C/mole amino acid).

AA^* = curies of exogenous ^{14}C-labelled amino acid added to each aliquot of perchlorate extract of tissue.

$\therefore AA^* = SAaa \times AAext$

AAint = moles of endogenous amino acid present in each aliquot of perchlorate extract of tissue.

SAdc = specific acitivity of dansyl chloride (curies ^{3}H/mole dansyl chloride)

N = number of d.p.m. per curie.

For the six amino acids studied (glutamate, aspartate, glutamine, hydroxyproline, GABA and glycine) the conditions of dansylation were such that the reaction between dansyl chloride and the amino acid was equimolar. Therefore considering a spot of a dansyl-amino acid which has been removed from a chromatogram and counted for ^{3}H and ^{14}C activity:-

Total no. of moles of amino acid present in spot (as dansyl derivative) $= \frac{^{3}H \text{ dpm}}{\text{SA dc}} \times \frac{1}{N}$

With respect to ^{14}C, the specific activity of the amino acid present in spot (as dansyl derivative)

$$= \frac{^{14}C \text{ dpm}}{\text{moles of amino acid in spot}} \times \frac{1}{N}$$

$$= \frac{^{14}C \text{ dpm}}{^{3}H \text{ dpm}} \times \text{SA dc}$$

However as explained earlier the specific activity (w.r.t. ^{14}C) of the amino acid in the spot (present as dansyl derivative) is the same as specific activity of the combined pool of endogenous and exogenous amino acid.

The specific activity of the combined pool of exogenous and endogenous amino acid $= \frac{AA^*}{AAint + AAext}$

$$\therefore \quad \frac{AA^*}{AAint + AAext} = \frac{^{14}C \text{ dpm}}{^{3}H \text{ dpm}} \times SAdc$$

$$\therefore \quad \frac{AAint}{AA^*} = \frac{^{3}H \text{ dpm}}{^{14}C \text{ dpm}} \times \frac{1}{SAdc} - \frac{AAext}{AA^*}$$

$$\therefore \quad AAint = AA^* \left\{ \left(\frac{^{3}H \text{ dpm}}{^{14}C \text{ dpm}} \times \frac{1}{SAdc} \right) - \frac{1}{SAaa} \right\}$$

Current method for the extraction, dansylation and quantitative estimation of free amino acids in rat cerebral cortex.

The scheme of the current method has been summarised in Fig. 3:7.

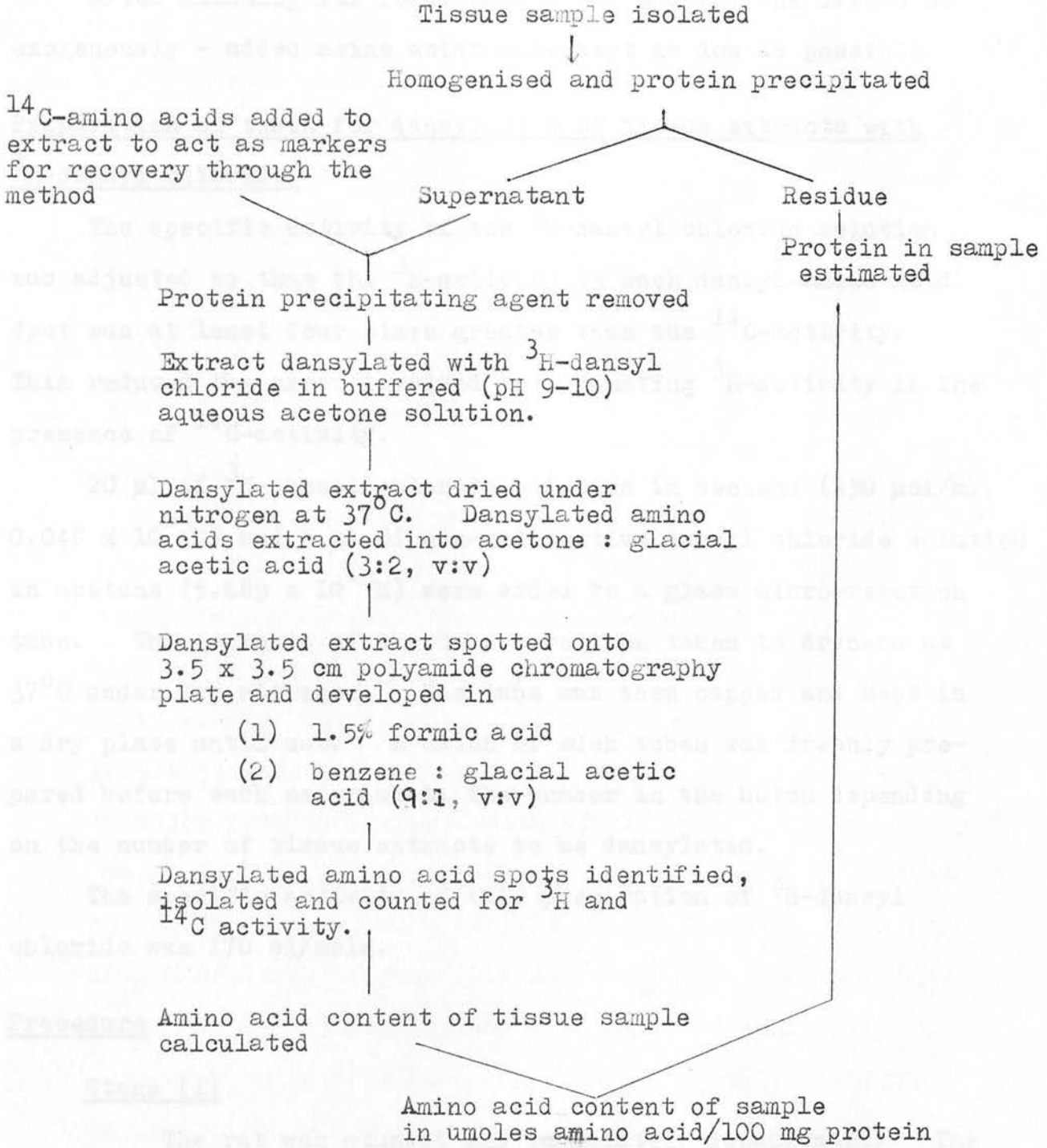
Equipment and materials - as described on pages 211 to 214, with the exception of the ^{3}H-dansyl chloride preparation which is described below.

Stock solution of ^{14}C-amino acids

The composition of this solution (shown on page 214), an aliquot (2 µl) of which was used as an internal standard in the dansylation procedure, was adjusted so that:-

(1) After dansylation and chromatography of a tissue extract (+ an aliquot of the ^{14}C-amino acid mixture) the dansyl-amino spots pertaining to these particular amino acids would be visible under u.v. light even if none of the amino acids were present in the tissue sample.

FIG. 3:7 General scheme for the quantitative estimation of amino acids in brain tissue by the dansylation method.



(2) the dansyl-amino spots contained sufficient ^{14}C activity for them to be readily counted.

After allowing for requirements (1) and (2) the levels of exogenously - added amino acids were kept as low as possible.

Preparation of tubes for dansylation of tissue extracts with ^{3}H-dansyl chloride.

The specific activity of the ^{3}H-dansyl chloride solution was adjusted so that the ^{3}H-activity in each dansyl-amino acid spot was at least four times greater than the ^{14}C-activity. This reduced the error involved in estimating ^{3}H-activity in the presence of ^{14}C-activity.

20 µl of ^{3}H-dansyl chloride solution in benzene (250 µci/ml, 0.048×10^{-3}M) and 5 µl of non-radioactive dansyl chloride solution in acetone (5.689×10^{-3}M) were added to a glass micro-reaction tube. The contents of the tube were then taken to dryness at 37°C under dry nitrogen. The tube was then capped and kept in a dry place until use. A batch of such tubes was freshly prepared before each experiment, the number in the batch depending on the number of tissue extracts to be dansylated.

The specific activity of this preparation of ^{3}H-dansyl chloride was 170 ci/mole.

Procedure

Stage (1)

The rat was stunned and immediately decapitated. The skull was rapidly opened and the cortex rapidly frozen

"in situ" with a dichlorodifluoromethane spray (Polar Spray, Medical Aerosols Ltd.). With a metal cork borer (diameter 3.5 mm) a small plug of frontal cortex was removed and the white matter trimmed off. The sample of cortical grey matter (between 20 and 30 mg wet weight) was immediately placed in a small plastic centrifugation tube (1.2 ml capacity, Eppendorff) containing liquid nitrogen. The liquid nitrogen was allowed to evaporate and the tube containing the now frozen tissue was placed in a deep-freeze at -20°C for 18 hours, until assay. This procedure took between two and three minutes.

Stage (11)

The tube containing the deep frozen sample of cortical tissue was removed from the deep-freeze. 200 µl ice-cold 0.48 M perchloric acid solution were added to the tube and the tissue was homogenised by hand for 5 minutes using a small ground glass pestle shaped to fit the tube. The homegenate was then allowed to stand at 4°C for 20 minutes to allow further fragmentation of tissue. The small polypropylene tube containing the homogenate was then centrifuged at 20,000 g for 20 minutes (2°C) using the high speed head of a Mistral centrifuge (M.S.E.). During centrifugation the small polypropylene tube was cushioned inside a polycarbonate centrifuge tube (capacity 7 ml) with tightly packed cotton wool. After centrifugation the supernatant

was carefully removed leaving a pellet of denatured protein in the bottom of the tube. The tube with the protein residue was deep frozen at -20°C until a later stage when the protein content was analysed.

Stage (III)

A 10 µl aliquot of the protein-free perchlorate extract was transferred to a small glass micro-reaction tube (description, page 211) to which was then added 2 µl of a stock solution containing known amounts of ^{14}C-labelled amino acids. (As listed earlier on page 214 this solution contained ^{14}C-glutamic acid, ^{14}C-aspartic acid, ^{14}C-glutamine, ^{14}C-GABA, ^{14}C-glycine and ^{14}C-hydroxyproline). 2 µl of 1.5 M potassium carbonate solution were added to the tube (with vigorous mixing) causing the evolution of CO_2 and the precipitation of perchlorate as its insoluble potassium salt. On a scaled-up experiment this addition of potassium carbonate adjusted the pH of the extract to 9.7. With the small volumes used in the actual assay it was not possible to measure the final pH but it was assumed to lie within the range 9.3 - 9.8. The extract was left to stand for 10 minutes at 4°C to assist the precipitation of potassium perchlorate (this salt is noticeably less soluble at low temperatures). The potassium perchlorate was then spun down by centrifuging the tube (placed inside a small polypropylene reaction tube (Eppendorff)) at 10,000 g for 2 minutes. 5 µl of the alkaline supernatant was then dansylated as described in Stage (IV).

Stage (IV)

5 µl of redistilled acetone were added to a pre-prepared (page 241) glass micro-tube containing ^{3}H-dansyl chloride (specific activity 170 ci/mole) and the tube was vigorously agitated (Whirlimix) to ensure that all the solid dansyl chloride was taken up into the acetone.

To this tube containing 5 µl of H^{3}-dansyl chloride (5.88×10^{-3}M)were then added 5 µl of the alkaline supernatant from the potassium perchlorate-precipitation step described in Stage (III). The tube contents were thoroughly mixed and the tube capped (with the rubber seal from the plunger of a 1 ml polyethylene syringe) and left in the dark at room temperature for 30 minutes. The contents of the tube were then taken to dryness (37°C) under dry nitrogen. At this point the tube could be capped and stored in the dark, in a dry atmosphere, until the chromatography step (Stage V).

Stage (V)

The dry contents of the micro-reaction tube were extracted with 10 µl acetone : glacial acetic acid (3:2, v:v) mixture. The tube was vigorously agitated and then centrifuged at 10,000 g for 2 minutes (micro-reaction tube placed inside an Eppendorff polypropylene reaction tube) to concentrate the undissolved salts at the bottom of the tube. Using a fine glass micro-capillary with a flamed tip a small

volume of the supernatant (between 0.1 and 0.2 µl) was carefully applied to the corner of a 3.5 x 3.5 cm polyamide plate (0.5 cm in from each edge). This procedure was carried out in the visual field of a stereo-microscope. A gentle flow of cold air was blown over the plate during the application of the acetone: glacial acetic acid mixture. With experience it was possible to tell when the spot was becoming saturated with salt. The application of material to the plate was stopped at this point (usually after the application of 0.1 to 0.2 µl).

Stage (VI)

The bottom of a micro-chromatography tank was just covered with 1.8 ml 1.5% (v/v) formic acid solution (solvent system 1). Using fine tweezers the prepared plate was then carefully lowered into the tank and developed in the ascending direction with the lid of the tank on. When the solvent front was within 3 mm of the top, the plate was removed and dried in a stream of warm air. It was then examined under short wavelength u.v. light. In the event of evidence of severe streaking (too much salt applied to plate), or insufficient fluorescence (too little of the dansyl derivatives applied to plate) the plate was discarded at this stage. If satisfactory (a large blue spot of dansyl hydroxide set two-thirds of the way along an almost continuous line of yellow-green dansyl amino acid spots), the plate was then run in

ascending fashion in solvent system (2) (benzene : glacial acetic acid, 9:1), at 90° to the direction of the first run. For the first 9 minutes of this run the lid of the tank was left off (see page 224). After this period the lid was placed on the tank and the solvent front run up to within 3 mm of the top of the plate. The plate was then removed from the tank, dried in a stream of warm air, and examined under u.v. light.

Under u.v. light the dansyl-GABA, dansyl-glycine, dansyl-glutamate, and dansyl-glutamine spots were very obvious by their strong fluorescence and characteristic positions on the plate (Fig. 3:6). The dansyl-hydroxyproline spot was very faint but it was always characteristically isolated from all other spots. Dansyl-aspartate formed a relatively faint spot between the prominent dansyl-glutamate and dansyl-hydroxide spots.

The spots required for counting were carefully ringed with a soft pencil. In the calculation of the endogenous amino acid levels the absolute activity of the spot was not used but rather the ratio of ^{3}H to ^{14}C activity. For this reason it was unnecessary to remove the whole spot for counting. Isolating and counting only the central intensely fluorescent zone of a dansyl-amino acid spot reduced the danger of contamination by neighbouring spots, thus increasing the specificity of the assay. A spot on the plate showing no fluorescence was ringed to serve as a background sample.

The marked spots were then carefully cut from the plate with a pair of fine scissors and placed in scintillation vials containing 1 ml ethanol. To assist the elution of the dansyl derivatives from the plate into the ethanol the vials were agitated vigorously for 10 seconds on a Whirlimix. 10 ml of toluene scintillant solution were added to the vials. These were then counted in a liquid scintillation counter Nuclear Chicago, Mark II) in two channels set to give maximum differentiation between ^{14}C and ^{3}H activity (Nuclear Chicago Mark II manual). The efficiency of counting in each of these channels was determined by an external standard quench correction procedure (Hetenyi and Reynolds, 1967).

The concentrations of endogenous amino acids in the perchlorate extract were calculated from the ratio of ^{3}H to ^{14}C activity in the dansyl-amino acid spots as described in the mathematical analysis of the technique (pages 236 to 239). By determining the amount of protein originally precipitated from the brain sample by the perchloric acid extraction, it was then possible to calculate the amino acid content of the sample in terms of μmoles amino acid per 100 mg protein.

In the technique which has been described it has been assumed that ^{14}C activity in the spots counted was specific to dansyl amino acids. The validity of this important assumption was proved by showing that ^{14}C amino acids them-

selves when chromatographed in solvent systems (1) and (2) ran at the first solvent front. There was therefore no possibility that the non-dansylated ^{14}C amino acids in the final mixture applied to the plate could have contaminated any of the dansyl-amino acid spots.

Estimation of protein in samples of brain tissue

Protein was estimated by the method of Lowry, Rosebrough Farr and Randall (1951).

Materials

0.75 N sodium hydroxide solution.

2% (w/v) sodium carbonate in 0.1 N sodium hydroxide solution.

2% (w/v) sodium potassium tartrate solution.

1% (w/v) copper sulphate solution.

Folin-Ciocalteau reagent (B.D.H.).

Stock solution of bovine serum albumin (Sigma) (2.5 mg/ml).

Distilled deionised water.

Initial preparation of samples and standards

The pellet of denatured protein remaining after the perchloric acid extraction of brain tissue (approximately 20 mg wet weight of brain) was solubilised in 1.0 ml 0.75 N sodium hydroxide solution. This process was accomplished in a period of one to two hours by placing the small polypropylene reaction tube (Eppendorff) in a shaker (Eppendorff). 0.1 ml of the alkaline protein digest (vigorously mixed just prior to sampling) was

diluted to 2.2 ml with water. The protein estimation was performed on a 0.1 ml aliquot of this dilution.

A stock solution of bovine serum albumin (2.5 mg/ml) was diluted to give standard protein solutions of 25, 50, 100, 150 and 200 µg/ml albumin. 0.1 ml aliquots of these solutions were used in the reaction.

Reaction

Reagent solution A was freshly prepared; i.e. 0.2 ml of a 2% solution (w/v) of sodium potassium tartrate and 0.2 ml of 1% (w/v) copper sulphate solution were added to 20 ml of a 2% solution (w/v) of sodium carbonate in 0.1 N sodium hydroxide.

0.5 ml of solution A was added in turn to : 0.1 ml of each of the 1/22 sample dilutions; 0.1 ml of each of the standard protein solutions; 0.1 ml of water. These tubes were allowed to stand for 10 minutes.

0.5 ml of freshly prepared solution B (1.0 ml Folin-Ciocalteau's reagent + 1.5 ml of water) was then added (with vigorous mixing - Whirlimix) to all the tubes in the assay, namely, the samples, the standards and the reagent blank. The tubes were then allowed to stand for 30 minutes.

After this period the optical density of the solutions at 750 mµ was read in a spectrophotometer (Perkin-Elmer) against the reagent blank. Over the standard range the optical densities of the final solutions were linearly related to their protein concentration. Appropriate dilution factors were used to calculate the amount of protein in the original sample of brain tissue.

Animal Methodology

The operative technique for the implantation of cobalt into the cortex of the rat, and the insertion of stainless steel screw electrodes into the skull, has been described in detail by Dow, McQueen and Townsend (1972).

I am very much indebted to Dr. J. K. McQueen and Mr. R. Dow for the preparation and electrocorticogram recording of the three "cobalt" rats used in this study.

Male Piebald Virol Glaxo (PVG) rats, approximately 220 g in weight (age 3 months) were anaesthetised with a halothane/oxygen mixture. A 4% halothane (Fluothane, I.C.I. Ltd.) mixture was used during induction while 2-2.5% mixture was used to maintain anaesthesia. A curved incision was made in the scalp, starting close to the left eye and extending 2-3 cm backwards. The soft tissues were retracted. The skull was trephined on both sides, 3 mm out from the midline, at points 3 mm anterior to, and 3 mm posterior to, the coronal suture (Fig. 3:8). These drill holes (2 mm diameter) allowed approach to the underlying cortex and prepared the skull for the insertion of the four stainless steel recording electrodes. The dura over the right frontal cortex was split with the tip of a 23 gauge needle. A cube of cobalt gelatin (1 mm^3), prepared as described by Fischer, Holubar and Malik (1967), was inserted vertically into the cortex so that its top was flush with the cortical surface. The specially-constructed, hollow, stainless-steel screws (8 BA, overall length 8 mm) which

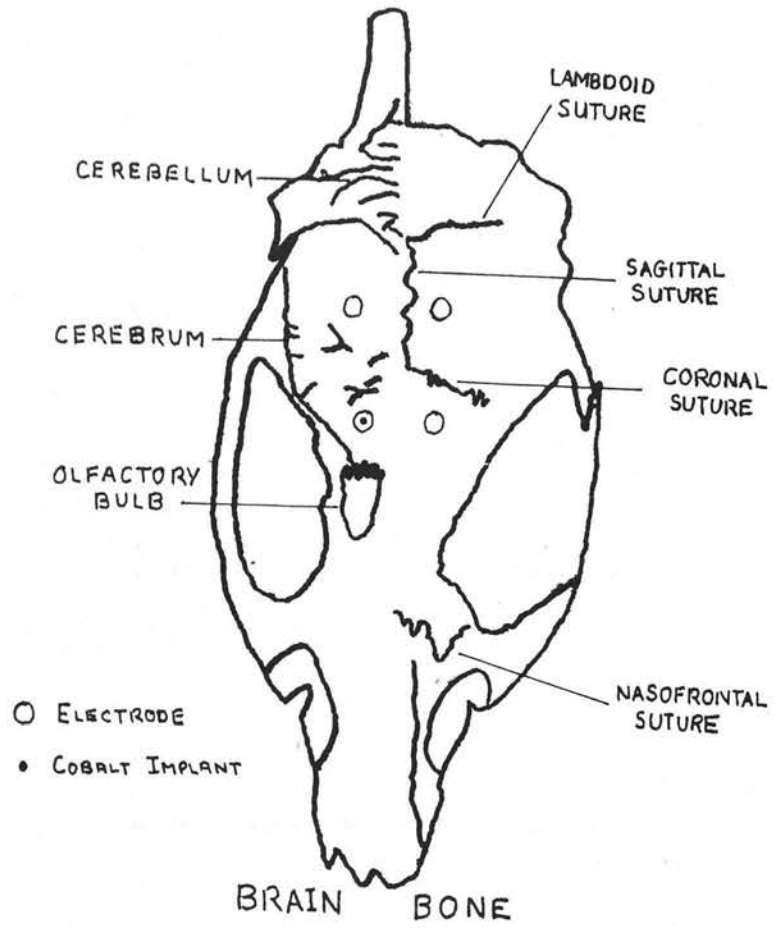


FIG. 3.8

Diagram of rat skull/brain showing the positions of the extradural recording electrodes and the cobalt implant.

served as extradural electrodes were then inserted into the four drill-holes. The collar of each screw was secured to the bone with cold curing acrylic resin (Simplex). Holes were then punched in the skin flap so that this could be fitted neatly around the electrodes. The incision was closed with Michel clips.

Electrocorticogram (ECoG) recordings were made from the unrestrained conscious rat (Fig. 3:9) within 24 hours of the operation. Spring connectors (Amphenol) fitted into the hollow screws allowed the brain potentials to be recorded on a Grass Model 7 polygraph. Fig. 3:10 shows an ECoG recording taken from one of the three cobalt rats at 11 days post-operation, one hour before it was killed. The ECoGs were similar in all the three animals. The record from the "cobalt" animal shows high amplitude left-sided polyspikes. An ECoG from a control animal is shown above the "cobalt" animal record.

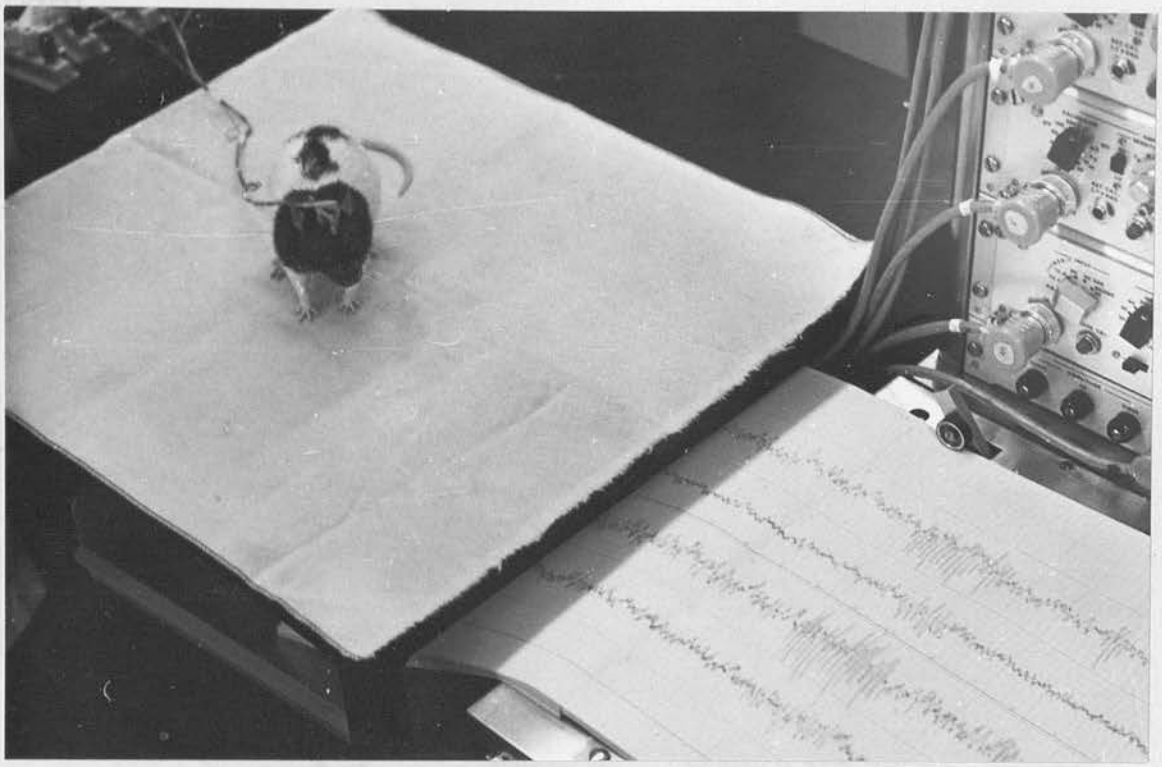


FIG. 3:9

Photograph showing the recording of a rat's electrocorticogram. The spring connectors attached to the recording leads have been inserted into the permanently implanted electrodes in the rat's skull. The Grass Model 7 Polygraph can be seen on the right of the picture.

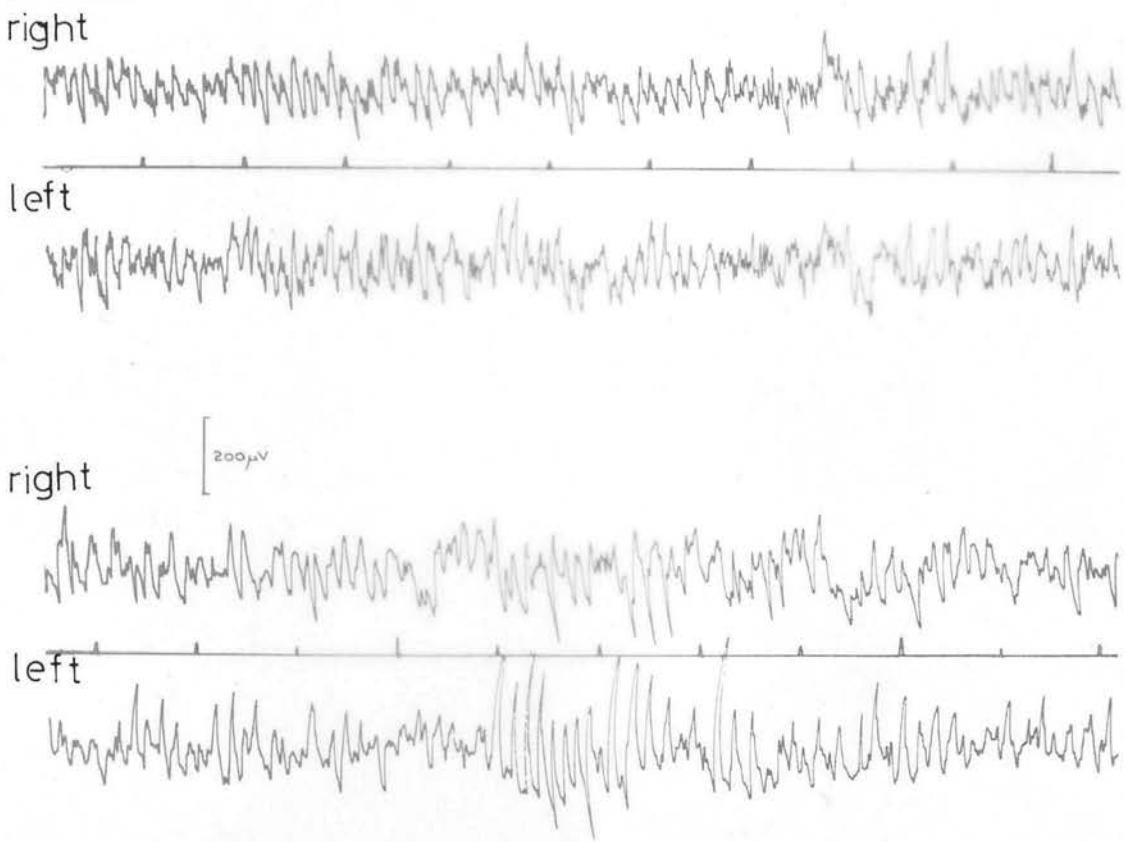


FIG. 3:10 Electrocorticogram (E Co G) recordings from control (top) and "cobalt" (bottom) rats.

RESULTS

Presentation of Glutamine Data

With the present solvent system it has not been possible to separate the dansyl derivatives of glutamine and threonine. It has been assumed that the recovery of threonine through the method is similar to the recovery of ^{14}C glutamine and on this basis I have calculated a composite figure expressing the sum of glutamine and threonine activity in terms of μmoles/100 mg protein.

McIlwain and Bachelard (1971) have collected together data on brain amino acids from a number of sources and it would appear from these data that the level of threonine in the brain is between 5% and 10% of the level of glutamine. The assumption, stated above, of a similar recovery for threonine to that of glutamine is thereforeunlikely to have led to serious errors in the estimation of their combined activities.

Amino acid analyses on the frontal cortices of control rats

Glutamate, GABA, glycine, hydroxyproline, aspartate and glutamine in combination with threonine, have been estimated in samples of frontal cortex from three rats. From the perchlorate extract of each cortical sample two separate aliquots were removed and analysed. A standard mixture of ^{14}C-amino acids was first added to each aliquot. The aliquots were then dansylated, and the dansyl derivatives separated by chromatography on polyamide plates. The spots representing the dansyl derivatives of

the amino acids under study were identified, and counted for ^{3}H and ^{14}C activity.

The results of the analyses are shown in Tables 3:4-6. Comparison of the ^{14}C activity recovered from each dansyl amino acid spot with the amount of each ^{14}C-amino acid taken through the dansylation procedure (Table 3:3) reveals that the final calculations have been based on the "recovery" of only a small fraction of the amino acids originally present. Three factors were responsible for this apparent low recovery.

Most important was the fact that only about 1-5% of the final dansylated extract was actually spotted onto a chromatogram (0.1-0.5 µl out of a volume of 10 µl). The actual volume spotted on has not been measured, because a knowledge of this volume is not essential in the calculations and the calibration of microcapillaries is a difficult and time-consuming task.

Another factor which tended to produce a low apparent recovery was the practice of partial removal of the dansyl-amino acid spots whereby only the central intensely-fluorescent zone of the spot was actually removed for ^{14}C and ^{3}H counting. This technique avoided contamination from neighbouring spots. In fact because of the generally excellent resolution of the spots (Fig. 3:6) and the small distances over which the dansyl derivatives were chromatographed the losses of dansyl amino acid in this way were normally very slight.

The final factor influencing the recovery of ^{14}C-amino acids

TABLE 3:3 : Exogenous ^{14}C-amino acids in that portion of each tissue extract which was actually dansylated.

	Specific activity of exogenously added ^{14}C-amino acid	Quantity† of exogenous ^{14}C-amino acid in each 5 µl aliquot of alkaline tissue extract prior to dansylation	
	(Ci/mole)	(nci)	(d.p.m.)
Glutamate	260	11.16	24775
Glutamine	45	8.37	18581
GABA	204	4.46	9901
Glycine	108	4.46	9901
Hydroxyproline	38	4.46	9901
Aspartate	227	8.93	19825

† These figures have been arrived at by multiplying the amounts present in a 2 µl aliquot of the stock solution of these ^{14}C-amino acids (page 214) by a factor of 5/14 to allow for the fact that only this proportion of each aliquot was actually taken through the dansylation step. The corrected figures shown constitute the AA* terms described in the mathematical analysis of the technique (page 237).

TABLE 3:4 : Analysis of selected amino acids in frontal cortex of Rat 1 (control)

	LEFT CORTEX Weight of protein in sample 2.024 mg				RIGHT CORTEX Weight of protein in sample 2.263 mg			
	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ ratio	Amino Acid(3) µmoles/100 mg protein	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ ratio	Amino Acid(3) µmoles/100 mg protein
Glutamate	242	13408	55.40	10.94	153	8919	58.29	10.44
	143	8263	57.78	11.41	167	11914	71.34	12.80
Glutamine +	253	10074	39.82	5.40	144	6428	44.64	5.55
Threonine	163	5898	36.18	4.86	207	9827	47.47	5.94
GABA	281	7356	26.18	2.02	119	3171	26.65	1.87
	179	4551	25.42	1.96	181	5524	30.52	2.15
Glycine	252	3183	12.63	0.88	118	1392	11.80	0.74
	171	1642	9.60	0.64	171	2133	12.47	0.79
Hydroxyproline	231	1082	4.68	0.02	137	627	4.58	0.01
	185	804	4.35	-0.01	225	947	4.21	-0.02
Aspartate	156	5504	35.28	5.52	60	2281	38.02	5.40
	84	1521	18.11	2.78	59	2245	38.05	5.41

(1) ^{14}C activity of dansylated amino acid spot.

(2) ^{3}H activity of dansylated amino acid spot.

(3) Refers to endogenous amino acid content of tissue sample.

TABLE 3:5 : Analysis of selected amino acids in frontal cortex of Rat 3 (control)

	LEFT CORTEX Weight of protein in sample 1.723 mg				RIGHT CORTEX Weight of protein in sample 2.044 mg			
	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ dpm	Amino Acid(3) µmoles/100 mg protein	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ dpm	Amino Acid(3) µmoles/100 mg protein
Glutamate	19	804	42.32	9.64	81	4548	56.15	10.98
	-	-	-	-	43	3401	76.09	15.52
Glutamine +	30	1012	33.73	5.20	66	2518	38.15	5.10
Threonine	24	869	36.21	5.63	78	3558	45.62	6.21
GABA	38	669	17.61	1.55	75	1804	24.05	1.84
	22	746	33.91	3.06	54	1598	29.59	2.27
Glycine	48	375	7.81	0.58	76	777	10.22	0.68
	32	438	13.69	1.12	76	971	12.78	0.89
Hydroxyproline	55	199	3.62	-0.08	75	322	4.29	-0.01
	67	334	4.99	0.05	124	538	4.34	-0.01
Aspartate	19	873	45.95	8.37	38	1026	27.00	4.16
	-	-	-	-	18	780	43.33	6.74

(1) ^{14}C activity of dansylated amino acid spot.

(2) ^{3}H activity of dansylated amino acid spot.

(3) Refers to endogenous amino acid content of tissue sample.

TABLE 3:6 : Analysis of selected amino acids in frontal cortex of Rat 5 (control)

	LEFT CORTEX Weight of protein in sample 2.768 mg				RIGHT CORTEX Weight of protein in sample 2.399 mg			
	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ ratio	Amino Acid(3) µmoles/100 mg protein	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ ratio	Amino Acid(3) µmoles/100 mg protein
Glutamate	107	9461	88.42	13.29	96	5897	61.43	10.43
	129	12464	96.62	14.53	78	5441	69.76	11.86
Glutamine +	174	9735	55.95	5.92	196	7776	39.67	4.62
Threonine	166	10734	64.66	6.91	118	4742	40.19	4.69
GABA	171	8434	49.32	2.93	188	5654	30.07	2.01
	121	7137	58.98	3.52	144	4963	34.47	2.31
Hydroxyproline	129	576	4.47	0	172	656	3.81	-0.05
	203	954	4.70	0.01	157	696	4.43	0
Aspartate	25	1202	48.08	5.73	68	2810	41.32	5.57
	66	2666	40.39	4.80	24	755	31.46	4.22

(1) ^{14}C activity of dansylated amino acid spot.

(2) ^{3}H activity of dansylated amino acid spot.

(3) Refers to endogenous amino acid content of tissue sample.

was the yield of the dansylation reaction. Apart from the incomplete dansylation of the amino acids (reaction I, Fig. 3:3), almost certainly some of the dansyl-amino acids formed were broken down through further attack by dansyl chloride (reaction III, Fig. 3:3). The improvements in the recovery of glutamate and aspartate which were brought about by reducing the time of dansylation and decreasing the concentration of dansyl chloride in the reaction mixtures suggest that dansyl-glutamate and dansyl-aspartate may be particularly vulnerable to attack by dansyl chloride.

In recovery experiments where I measured the volume of the final dansylation mixture applied to a plate the yield of dansylated-GABA, glycine, glutamine and hydroxyproline was between 40 and 90%. Glutamate and aspartate gave a 10-40% yield of dansylated product (Table 3:2).

Estimation of aspartate in brain tissue

The dansylation techniques were not entirely satisfactory for aspartate. The agreement between duplicate estimates was not always good for this amino acid. Two factors would seem to have contributed to the variability of the aspartate results.

Firstly, under the present experimental conditions the dansylation of aspartate gave a poor yield of dansylated product (Table 3:2).

Secondly, the dansyl-aspartate spot was very close to the large dansyl-glutamate spot. Almost certainly the dansyl-

aspartate spot on some occasions was contaminated with dansyl-glutamate.

Because of the degree of variability of the aspartate results they were not included in the analysis of the data. In order to assay aspartate levels satisfactorily one would have to prepare a separate chromatogram for this amino acid and continue the extended run in solvent system (2) until the dansyl-aspartate was clearly separated from dansyl-glutamate.

Hydroxyproline

As can be seen in Table 3:5 the analysis of hydroxyproline sometimes gave a negative figure for the level of this amino acid in cortical tissue. Hydroxyproline is an important constituent of collagen and gelatin but no reference could be found describing its detection as a free form in brain. If it was present in the cortical tissue of the rats it was below the detection limits of the dansylation technique. In these studies therefore the variance of the hydroxyproline results about zero gave an estimate of the variance of the method.

Glutamate, glutamine and threonine, GABA and glycine levels in the frontal cortices of control rats.

These amino acids were satisfactorily measured by quantitative dansylation techniques. The agreement between independent estimates (independent at least after the initial homogenisation step) on the same tissue sample was good (Table 3:7).

TABLE 3:7 : Amino acid levels (umoles/100 mg protein) in rat frontal cortex (control rats).

Rat		1.		3.		5.	
Side of brain		L	R	L	R	L	R
Glutamate		10.94	10.44	9.64	10.98	13.29	10.43
		11.41	12.80	-	15.52	14.53	11.86
	$\bar{x}$*	11.2	11.6		13.3	13.9	11.2
Glutamine + Threonine		5.40	5.55	5.20	5.10	5.92	4.62
		4.86	5.94	5.63	6.21	6.91	4.69
	$\bar{x}$	5.13	5.75	5.42	5.66	6.42	4.66
GABA		2.02	1.87	1.55	1.84	2.93	2.01
		1.96	2.15	3.06	2.27	3.52	2.31
	$\bar{x}$	1.99	2.01	2.31	2.06	3.23	2.16
Glycine		0.88	0.74	0.58	0.68	0.91	0.71
		0.64	0.79	1.12	0.89	0.83	0.76
	$\bar{x}$	0.76	0.77	0.85	0.79	0.87	0.74

Footnote:* $\bar{x}$ is the mean of the duplicate estimates.

As an index of the likely error limits of the technique, the deviation of the duplicate estimates from their mean has been calculated for each amino acid. The results of this analysis are summarised in Table 3:8. This analysis showed that for these four amino acids the technique has a good degree of reproducibility (average deviation approximately ± 5%).

Because the variation between the mean amino acid levels in the left and right cortices was greater than the variation between the mean cortical levels in the three rats (i.e. between cortices variation greater than between rat variation), the results from the left and right cortices of the three rats have been treated as independent estimates of the mean amino acid content of rat cortical tissue. Control amino acid levels in rat cortical tissue, as determined in the present studies, are shown in Table 3:9, along with previously published values.

In many previous studies of brain amino acids the results have been expressed in terms of micromoles of amino acid per gram of tissue weight. There are however considerable difficulties in weighing small amounts of frozen tissue, both in respect of getting an accurate figure and in keeping the tissue frozen. In this present study therefore the level of free amino acids in each tissue sample has been related to the weight of protein in the sample.

Battistin, Grynbaum and Lajtha (1969) found that 1 gram (wet weight) of cortical tissue contained 83.5 ± 4.0 (mean ± s.d.)

TABLE 3:8 : Variability of Assay Technique

	% Variation* of duplicate estimates
Glutamate	5.8 ± 4.9 % (11)
Glutamine + Threonine	4.8 ± 3.5 % (11)
GABA	6.3 ± 5.4 % (11)
Glycine	4.6 ± 5.2 % (11)

* Mean deviation of the individual values of duplicate estimates from the mean of the duplicates, expressed as a percentage of that mean; mean ± S.D. (no. of duplicates).

The assays on the left cortex of rat 3 have been excluded from this analysis because neither chromatogram was satisfactory.

TABLE 3:9 : Amino acid content of rat cortex : comparison between present data, obtained by use of dansylation techniques, and previous data obtained by other chromatographic or enzymic methods.

	Present Study µmoles/100 mg protein	Present Study µmoles/g wet weight[3]	Berl and Waelsch(1958) µmoles/g wet weight	Okumura, Otsuki and Aoyama(1959) µmoles/g wet weight	Kandera, Levi and Lajtha(1968) µmoles/g wet weight
Glutamate	11.8[1] ± 1.6(6)	10.2	10.4 - 12.5	10.0	11.6
GABA	2.29 ± 0.47(6)	1.98	1.5 - 2.7	2.14	2.33
Glycine	0.80 ± 0.05(6)	0.69	-	0.91	0.63
Glutamine[2] Threonine	5.51 ± 0.60(6)	4.77	4.6 - 5.4	4.8 0.30	2.35[4]

[1] mean ± s.d. (no. of estimates)

[2] bracket denotes a combined value for glutamine + threonine

[3] data in column 1 multiplied by conversion factor of 0.865 - see text page 264 for explanation.

[4] some destruction of glutamine during column chromatography at 60°C.

mg protein. The data of Fahn and Côté (1968) indicate a slightly higher figure of 89.5 mg for the protein content of 1 gram of cortical tissue. On this basis 100 mg of protein would therefore be "equivalent" to between 1.05 and 1.20 g of cortical tissue (wet weight).

To allow direct comparison between the present data expressing amino acid content of tissue in terms of μmoles per 100 mg protein and previously published amino acid data in terms of μmoles per g wet weight tissue, the present data have been multiplied by a factor of 0.865 to convert to μmoles amino acid per gram wet weight. This conversion factor was based on a mean figure for the relationship between wet weight of tissue and protein content taken from the studies of Fahn and Côté (1968) and Battistin et al. (1969). Study of Table 3:9 reveals that the present data are entirely comparable with previously published data for amino acid levels in rat cortex.

Amino acids in the primary and secondary foci of rats with a cobalt-induced epileptogenic lesion.

Tables 3:10-12 show the amino acid analyses from the frontal cortices of rats which 9 days previously had cobalt implanted into the right frontal cortex. The sample of cortex taken on the right side was centred on the site of the cobalt implant and was considered to be a sample from the primary epileptic focus. A sample was taken from the corresponding area of the left frontal cortex. This was the site of the secondary "mirror" focus

TABLE 3:10 : Analysis of selected amino acids in frontal cortex of Rat 2 (cobalt implant)

	LEFT CORTEX Weight of protein in sample 1.443 mg				RIGHT CORTEX Weight of protein in sample 2.829 mg			
	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ dpm	Amino Acid(3) µmoles/100 mg protein	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ dpm	Amino Acid(3) µmoles/100 mg protein
Glutamate	34	875	25.74	6.84	51	4368	85.65	12.59
	70	1521	21.73	5.74	64	6135	95.86	14.10
Glutamine +	111	4692	42.27	7.87	62	3747	60.44	6.29
Threonine	86	4049	47.08	8.85	85	6317	74.32	7.84
GABA	118	1206	10.22	1.02	86	3288	38.23	2.21
	153	1879	12.28	1.25	52	2826	54.35	3.17
Glycine	103	1322	12.83	1.23	81	1078	13.31	0.69
	125	1537	12.30	1.17	79	1049	13.28	0.69
Hydroxyproline	101	424	4.20	-0.03	98	359	3.66	-0.05
	153	700	4.58	0.01	137	707	5.16	0.04
Aspartate	24	539	22.46	4.74	34	1812	53.29	6.23
	-	-	-	-	23	649	28.22	3.26

(1) ^{14}C activity of dansylated amino acid spot.

(2) ^{3}H activity of dansylated amino acid spot.

(3) Refers to endogenous amino acid content of tissue sample.

TABLE 3:11 : Analysis of selected amino acids in frontal cortex of Rat 6 (cobalt implant)

	LEFT CORTEX Weight of protein in sample 0.991 mg				RIGHT CORTEX Weight of protein in sample 2.167 mg			
	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ dpm	Amino Acid(3) µmoles/100 mg protein	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ dpm	Amino Acid(3) µmoles/100 mg protein
Glutamate	120	1888	15.73	5.87	95	6014	63.31	11.80
	88	1551	17.63	6.61	137	8626	62.96	11.73
Glutamine +	182	5632	30.95	7.94	142	7389	52.04	6.82
Threonine	198	6791	34.30	8.92	85	4333	50.98	6.67
GABA	134	1039	7.75	1.08	152	5187	34.13	2.51
	216	1759	8.14	1.14	198	6760	34.14	2.51
Glycine	125	1369	10.95	1.46	178	2058	11.56	0.75
	145	1585	10.93	1.46	315	3907	12.40	0.81
Hydroxyproline	89	452	5.08	0.09	152	657	4.32	-0.01
	135	650	4.81	0.05	175	791	4.52	0
Aspartate	31	127	4.10	1.04	31	928	29.94	4.40
	-	-	-	-	40	1021	25.53	3.73

(1) ^{14}C activity of dansylated amino acid spot.

(2) ^{3}H activity of dansylated amino acid spot.

(3) Refers to endogenous amino acid content of tissue sample.

TABLE 3:12 : Analysis of selected amino acids in frontal cortex of Rat 4 (cobalt implant)

	LEFT CORTEX Weight of protein in sample 2.003 mg				RIGHT CORTEX Weight of protein in sample 2.713 mg			
	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ dpm	Amino Acid(3) µmoles/100 mg protein	^{14}C(1) dpm	^{3}H(2) dpm	$^{3}H/^{14}C$ dpm	Amino Acid(3) µmoles/100 mg protein
Glutamate	93	5783	62.18	12.42	101	6302	62.40	9.50
	100	6424	64.24	12.84	54	3448	63.85	9.72
Glutamine +	156	7655	49.07	6.86	127	12466	98.16	10.89
Threonine	119	5733	48.18	6.72	58	5450	93.97	10.40
GABA	122	4062	33.30	2.62	117	5469	46.74	2.82
	151	5101	33.78	2.66	110	4828	43.89	2.65
Glycine	165	1735	10.52	0.72	172	2819	16.39	0.91
	142	1540	10.85	0.75	88	1503	17.08	0.95
Hydroxyproline	209	1061	5.08	0.05	156	795	5.10	0.04
	92	436	4.74	0.02	127	1192	9.39	0.30
Aspartate	44	2172	49.36	7.85	68	1087	16.99	1.88
	63	1140	18.10	2.80	25	555	22.20	2.64

(1) ^{14}C activity of dansylated amino acid spot.

(2) ^{3}H activity of dansylated amino acid spot.

(3) Refers to endogenous amino acid content of tissue sample.

(Dow, McQueen and Townsend, 1972).

As in the control studies there was good agreement between the two estimates of each amino acid in each tissue sample. Because of the small number of animals involved (3) and the necessary differentiation between the results from the left and right cortices, the data has been analysed in terms of individual values. From the data on control rats (Table 3:9) fiducial limits were calculated for the amino acid content of rat frontal cortex.

i.e.

$$\text{Amino acid content of rat (control) frontal cortex} = \left\{\begin{matrix}\text{mean estimate} \\ \text{from sample}\end{matrix}\right\} \pm (t) \left\{\begin{matrix}\text{standard} \\ \text{deviation of sample}\end{matrix}\right\}$$

where t is the "t" value at any specified level of probability.

On this occasion the sample was compared at six values, therefore "t" had five degrees of freedom.

Once the fiducial limits had been calculated for given levels of probability, the individual values from the "cobalt" rats were compared with these limits to see whether they were inside or outside the range of concentrations expected in control animals. The results of this analysis are shown in Table 3:13.

It can readily be seen that rats 2 and 6 showed an identical series of amino acid changes in the area of the secondary epileptic focus. There were significant increases in glycine

TABLE 3:13 : Amino acid levels (umoles/100 mg protein) in primary (right side) and secondary (left side) epileptic foci of rats with a cobalt-induced epileptogenic lesion in the right frontal cortex : comparison with levels in cortical tissue from control rats.

	Control	Rat 2("cobalt")		Rat 4("cobalt")		Rat 6("cobalt")	
Side of cortex		left	right	left	right	left	right
Glutamate	11.8[1] ± 1.6(6)	[6.29][2] ↓↓	13.4	12.6	9.61	[6.24] ↓↓	11.8
Glutamine + Threonine	5.51 ± 0.60(6)	[8.23] ↑↑	7.07	6.79	[10.6] ↑↑↑	[8.43] ↑↑	6.75
GABA	2.29 ± 0.47(6)	[1.14] ↓	2.69	2.64	2.74	[1.11] ↓	2.51
Glycine	0.80 ± 0.05(6)	[1.20] ↑↑↑	0.69	0.74	[0.93] ↑	[1.46] ↑↑↑	0.78

[1] mean ± s.d. (no. of estimates)

[2] boxed values indicate those which are different from control values
the number of arrows after the boxes indicate the significance of the difference.
one arrow $p < 0.10$
two arrows $p < 0.05$
three " $p < 0.005$
four " $p < 0.001$
an upward arrow indicates a value higher than control, a downward arrow a lower value than control.

($p < 0.001$) and glutamine/threonine ($p < 0.005$). Glutamate and GABA both tended to be lower. In neither of these two rats were any changes observed in the area of the primary focus.

In rat 4 on the other hand there was a significantly increased glutamine/threonine level ($p < 0.001$) and an increased glycine level ($p < 0.05$) in the primary focal area with no changes in the secondary focal area.

There was no satisfactory explanation why rats 2 and 6 displayed a different spectrum of amino acid changes from rat 4. The ECoG's recorded from each rat an hour before they were killed showed epileptiform spikes. The vast majority of the discharges arose from the secondary focus. These were associated with twitching of the vibrissae. Those spikes which were seen on the primary side always occurred in synchrony with discharges from the secondary focal area. There was no difference in the manner of killing, or in the sampling of brain tissue from, the three animals.

Because of the small numbers of animals studied (3) and the lack of a consistent pattern of amino acid changes in the three animals it has not been possible to draw any firm conclusion regarding the effect of the cobalt implant in the cortex. There was however evidence of major changes in the levels of glutamate, glutamine/threonine, glycine and GABA, particularly in the region of the secondary epileptic ("mirror") focus. It is highly unlikely that the observed changes arose by chance. Thus it would seem that epileptic processes are associated with disturbances of certain amino acids.

DISCUSSION

The study reported here would seem to establish the application of dansylation techniques to the quantitative microanalysis of tissue amino acids. The evidence for the success of the method lies firstly in the good agreement between duplicate estimates from the same tissue sample and secondly in the fact that the data from the control rats are comparable to previously published data for cerebral amino acids (see Table 3:8). Although aspartate was not satisfactorily estimated in this particular study there seems no reason why the techniques evolved should not be applicable to many other amino acids (Fig. 3:4).

There have been several previous reports of semi-quantitative amino acid analyses by dansylation techniques. In the first of these Crowshaw, Jessup and Ramwell (1967) dansylated extracts of cortical superfusate, chromatographed the products, eluted the separated dansyl-amino acid spots and assayed the fluorescence of the eluates. The fluorescence of the eluates was compared with the fluorescence of known amounts of dansyl-amino acid standards which were chromatographed, separated, eluted and assayed in a similar manner. The method gave no indication of the recovery of amino acids through the actual dansylation process. A similar shortcoming is to be found in the study of Neuhoff and Weise (1970), in which the final dansylated products were estimated by a radioactive method.

Briel, Neuhoff and Maier (1970) have recently published a comprehensive report on their studies of the analysis of tissue amino acids using dansylation techniques. Their method however requires very strict control of reaction conditions and an extrapolation from standard calibration curves. Also, the estimation of any one amino acid requires that all the dansyl derivatives in the mixture be estimated. The method is therefore highly involved, requires strict control of reaction conditions and makes too many assumptions to be termed quantitative.

The techniques outlined here therefore represent the first reliable use of dansylation to calculate absolute levels of amino acids in tissue. However there does remain room for investigation and further improvement of the present method.

In the present study ^{14}C-amino acid recovery standards were not added until after the protein precipitation step. It was assumed that there were no serious amino acid losses during protein precipitation. This may have introduced a slight error which would tend to underestimate the values for tissue amino acid content. However in many other amino acid studies it has been standard practice to assume complete extraction of amino acid from tissue (e.g. Okumura, Otsuki and Aoyama 1959; Johnson and Aprison, 1971). Also it has been shown previously (Yates, 1967) that addition of exogenous standards to brain homogenate prior to perchloric acid extraction may result in a variability in the recovery of the standard which is not a reflection of the recovery of the endogenous material. In future studies where it is intended

to work with smaller volumes of tissue the ^{14}C-amino acid standards will be added before the homogenisation and this problem will be examined with reference to these particular amino acids.

Perhaps the greatest scope for improving the method lies in the final chromatography stage. At present because of problems of loading the polyamide plate with inorganic salts only about 1-2% of the final dansylated extract (0.1 - 0.2 µl from 10 µl) can be actually chromatographed. If a way could be found of extracting the dansylated amino acids from the final solution, the sensitivity of the technique would be greatly increased.

As a first step towards a lower salt content in the final extract it might be possible to use a volatile protein precipitant. The fact that an agent such as acetone or ethanol can be completely removed from the solution after the protein precipitation step may outweigh the disadvantage of less efficient precipitation than that achieved by perchloric acid.

It would also be extremely useful to investigate further solvent systems and chromatography conditions which would allow the separation of dansyl-glutamine from dansyl-threonine.

Amino acid results

Glutamine/Threonine

The fact that the sum total of these two amino acids was altered significantly in the area of the secondary epileptic focus in two out of three rats makes it particularly important to find

separation techniques for the dansyl derivatives of these two amino acids. At present one can only speculate as to which of the two amino acids were in fact increased in the secondary focal area of the "cobalt" rats.

Threonine is an essential amino acid which cannot be synthesised by mammals. As far as is known, its principal role in metabolism is to serve as a constituent of protein. The breakdown products of threonine enter the general metabolic pools of acetyl-CoA and pyruvate. There is also a pathway converting threonine to glycine.

In contrast to threonine, glutamine is a non-essential amino acid being readily synthesised from glutamic acid and ammonia. It is a constituent of protein, and has an important function as a source, or acceptor, of nitrogen in many transamination reactions.

The low levels of threonine in rat brain (Table 3:9) and the fact that it cannot be synthesised by the rat suggest that major changes in the brain content of this amino acid would be unlikely unless there was considerable catabolism of protein in which case all free amino acid levels would be raised. The significant changes in the glutamine/threonine levels in the "cobalt" rats have therefore been attributed to glutamine.

Measurement of GABA in Brain Tissue

In the estimation of GABA levels in the brain one might be well advised to invoke the Heisenberg uncertainty principle, since

in obtaining a sample of tissue for biochemical analysis one is forced to alter the physiological state of the tissue. Such procedures as decapitation or immersion in liquid nitrogen are almost certain to induce intense, if only momentary, electrical activity in the brain. If indeed GABA is a transmitter substance then one might well expect such activity to lead to an alteration in its level at these sites where it has a transmitter function. One cannot even predict whether levels obtained with the use of any one particular method of killing the animal are any more representative of the "normal" physiological levels than levels obtained with any other method of killing.

Lovell Elliot and Elliot (1963) were the first to study this problem in detail. They found that rats killed by immersion in liquid air had a cerebral GABA level of 1.64 µmoles/g wet weight. Rats killed by decapitation ~~of~~ with the head falling straight into liquid air had a cerebral GABA level of 1.78 µmoles/g wet weight. When the rats were decapitated and the brains left at room temperature for 2 minutes before homogenisation in ethanol the GABA level rose to 2.39 µmoles/g wet weight. Minard and Mishawer (1964) have reported that after the first two minutes the post-mortem rise in GABA levels slows down considerably. They suggested, on the basis of their glutamate and GABA data, that the immediate post-mortem increase was due to continued conversion of a small highly active "pool" of glutamate to GABA.

Qualitatively similar post-mortem changes in cerebral GABA levels have been observed in human studies. A comparison of amino acids in autopsy tissue (Perry, Berry, Hansen, Diamond and Mok, 1971) ~~which~~ and amino acids in biopsy tissue (Perry, Hansen, Berry, Mok, and Lesk, 1971) demonstrated significant post-mortem increases in many amino acids including glutamate, glycine and GABA. It is interesting that the GABA and glutamate levels (0.42 and 5.96 μmoles/g wet weight cortex) reported for the biopsy samples fall well below the range of values collected together by McIlwain (1971) from a variety of studies in different species. This may be significant in that only a few seconds elapsed between removal of the biopsy sample and immersion in liquid nitrogen.

It can be seen therefore that measurement of GABA in cerebral tissue is not a straightforward operation. When looking for differences between control and treatment animals one must standardise the conditions of sampling. In this particular project I was interested in isolating and analysing a small amount of cortical tissue (20-30 mg). For this reason I did not consider freezing the decapitated head before removing the samples of cortical tissue since deep-freezing a head in liquid nitrogen makes subsequent isolation and removal of a particular brain area a difficult operation. However before carrying out more extensive follow-up studies on amino acids in "epileptic" tissue I shall require to develop techniques which will allow the accurate sampling of brain tissue under conditions which preclude or minimise continuing metabolism in the tissue.

Studies on epileptogenic tissue

In this study three rats with 11 day-old cobalt-induced epileptogenic lesions were sacrificed and analyses were made on the levels of glutamate, glutamine and threonine, GABA and glycine in both the primary and secondary foci. The fact that only three such rats were examined, and the fact that two of these rats showed amino acid changes very different from those observed in the third rat (without any electro-corticographic evidence of a marked difference between the electrical disturbances produced by the lesions) make it very difficult to draw any firm conclusion about the effect of the cobalt lesion. Nevertheless the similarity of the pattern of amino acid changes seen in rats 2 and 6 on the side contralateral to the lesion (an increase in glutamine and glycine levels, a decrease in glutamate and GABA levels) suggest that the generation of an epileptic focus in neural tissue may involve changes in amino acid metabolism.

It was perhaps rather surprising to find no significant changes at the site of the primary focus (except for a raised glutamine level inrat 4), particularly since associated with the cobalt lesion there is marked gliosis and capillary proliferation in the adjacent tissue (McQueen - unpublished results). However it should be noted that at the 11 day stage epileptiform discharges from the primary site were less, both in number and amplitude, than those from the contralateral "mirror" site. If the results observed here in two of the "cobalt" rats (rats 2 and 6) were to be repeated in further experiments, one might speculate

that the establishment of a secondary epileptic focus was associated with alterations in either the synthesis, storage or utilisation of certain amino acids.

With reference to changes in the tissue levels of glutamate and GABA it should be noted that glutamate and GABA are formed principally by operation of the "GABA shunt" in which α-keto-glutarate $\longrightarrow$ glutamate $\longrightarrow$ GABA $\longrightarrow$ succinic semialdehyde $\longrightarrow$ succinate $\longrightarrow$ citric acid cycle. It has been estimated (Balázs, Machiyama, Hammond, Julian and Richter, 1970) that 8% of the general oxidative metabolism of neural tissue may be by way of the "GABA shunt". One can radily see therefore that quite apart from any postulated role as neurotransmitters, GABA and glutamate are very important metabolic intermediates. Any alterations in the levels of these compounds in tissue therefore may not necessarily reflect increased or decreased transmitter release but rather a general alteration in tissue metabolism.

In fact however, while one may speculate whether decreased GABA and glutamate levels mean decreased availability of these compounds, or increased turnover with a resetting of the tissue stores at a lower level; for further interpretation of the meaning of the absolute tissue levels one requires some idea of the dynamics of the system. For this reason in further studies on epileptogenic tissue it would seem essential to carry out precursor labelling experiments which might give a clue as to the rate of turnover of these amino acids. The most one can say at present is that the observed changes in GABA and glutamate levels in

epileptogenic tissue might be related to the neurotransmitter roles postulated for those compounds (review; Krnjevic, 1970; Johnson, 1972).

It is tempting to link the increase of glutamine levels in the region of the secondary epileptic focus in two of the "cobalt" rats with the concomitant decrease in glutamate levels. This link seems particularly attractive in view of the report by Berl and Clarke (1969) that uptake of glutamate from the intraneuronal space is associated with glutamine synthesis. Watkins (1972) has hypothesised that the enzyme glutamine synthetase may be located principally in glial cells and post-synaptic neuronal elements. A very naive and completely speculative explanation of the increased glutamine levels and decreased glutamate levels in the secondary epileptic focus would be that there is an increased release of the excitatory transmitter glutamate, associated with increased uptake and glutamine synthesis by the post-synaptic elements. In the present state of knowledge however it would be difficult even to test such a hypothesis.

The increase in the glycine level in the secondary focus of two of the "cobalt" rats might be indicative of increased protein synthesis at this site since it has been shown that glycine levels in the brain are highest while the brain is actively maturing (Agrawal, Davis and Himwich, 1966; Bayer and McMurray, 1967; Levi and Morisi, 1971). However an investigation of the rates of protein synthesis in the primary and secondary epileptic

foci of these rats found no difference between control and "cobalt" rats (Dewar, Dow and McQueen, 1972).

Since the amino acid studies reported here were envisaged there have been two other reports on amino acid levels in epileptogenic cortical tissue.

Van Gelder, Sherwin and Rasmussen (1972) have reported amino acid analyses on samples of "focal epileptic" tissue from the frontal or temporal lobes of cases of human focal epilepsy. The region which demonstrated most epileptiform activity was sampled, as well as, whenever possible, an area of cortex far-removed from the focal area. They reported that there was a generalised decrease in the levels of GABA and aspartate throughout the cerebral cortex of these patients. It is not clear however how they arrive at this conclusion since the only control human data they quote (Perry et al., 1971) showed very much lower GABA and aspartate levels in biopsy samples of cerebral cortex. The results of Van Gelder et al. (1972) do suggest that "focal epileptic" tissue has a lower content of glutamic acid and taurine and a very much higher content of glycine than "control cortical" tissue from the same patient. The authors suggested that their findings indicated an uncoupling between glucose oxidation and anino acid metabolism in epileptogenic tissue.

The studies reported by Koyama (1971) are more comparable to our own in that he examined amino acid changes in cortex 24 hours after the application of cobalt powder to exposed pre-cruciate

cortex of the cat. At that time these cats with cobalt-induced epileptogenic lesions displayed clonic convulsions affecting all four extremities. In the tissue immediately caudal to the primary lesion site Koyama observed a 79% decrease in the GABA level, a 54% decrease in the glutamate level, and a 45% increase in the glycine level. The changes observed in the corresponding area of the contralateral cortex were a 56% decrease in the GABA level, a 10% decrease in the glutamate level and a 15% increase in the glycine level. Glutamine, threonine and aspartate levels were unchanged in both areas of cortex. In a qualitative sense these changes (apart from the lack of change in glutamine levels) resemble the changes seen in the secondary epileptic foci of rats 2 and 6 of the present study.

Several factors may have contributed to the rather different results of my own and Koyama's study. In the first place a different species of animal was used, and in the second place a different method of cobalt application was used to produce the epileptogenic lesions. The lesions and their effects may therefore have been radically different in the two studies. For instance in the present study one never saw the clonic convulsions affecting all four limbs which have been reported by Koyama. In the rats the characteristic seizure pattern was intermittent forelimb and whisker twitching. A further important factor which varied in the two studies was the time after cobalt treatment at which the amino acid analyses were carried out; it being 24 hours

in the case of the cats and 11 days in the case of the rats. For all these reasons it would perhaps be naive to expect to find identical amino acid changes in the two studies.

The important point to emerge from these three studies (Koyama, 1971; Van Gelder et al., 1972; present study, 1972) is that despite the differences in the form of epilepsy studied there was evidence that the levels of certain amino acids were altered by epileptic processes.

The development and further refinement of the present analytical technique for quantitative micro-determination of the amino acids involved in these processes may signal a new phase in the elucidation of the basic biochemical mechanisms which are concerned in the development of epilepsy.

SUMMARY

A new isotope dilution assay technique was developed for the micro-determination of GABA and associated amino acids in sub-milligram amounts of brain tissue. The method depends on the preparation, separation and measurement of the dansyl derivatives of the free amino acids in a perchlorate extract of brain tissue.

Values for GABA, glutamate, glutamine/threonine and glycine levels in rat cortex obtained by use of this technique were highly reproducible and in good agreement with previously published values.

These amino acids were studied in tissue samples from the primary and secondary epileptic foci of rats with an 11 day-old cobalt-induced epileptogenic lesion in the right frontal cortex. There was evidence that the levels of GABA and glutamate were lowered, and the levels of glycine and glutamine/threonine raised, in the region of the secondary epileptic "mirror" focus.

It was concluded that epileptic processes may be associated with disorders in amino acid metabolism.

The general problems of amino acid studies in the brain are discussed as is the significance of the findings in the rats with the cobalt-induced epileptogenic lesions.

GENERAL DISCUSSION

This short closing discussion will attempt to consider the findings of the three investigations against the general background of epilepsy and indicate further lines of research arising from them.

The important and surprising finding of the folate studies was that large oral doses of folic acid do not have any effect on the level of folate activity in the c.s.f. If, as seems reasonable, this can be taken as evidence for unaltered levels of folate activity in the central nervous system after oral folic acid, then Reynold's hypothesised link (Reynold's et al., 1966) between the folate-depleting effects of certain drugs (diphenylhydantoin, phenobarbitone and primidone) and their anticonvulsant action must be seriously challenged. A major argument in favour of this link was that folic acid replacement therapy in patients on chronic anticonvulsant treatment occasionally caused an increase in the severity and frequency of fits. The present findings argue against this being a direct "convulsant" effect of folic acid but rather support the contention that this effect results from more efficient metabolism of the anticonvulsant drug leading to subtherapeutic plasma concentrations of the anticonvulsant drug.

The other major argument in Reynold's hypothesis was that prolonged treatment of epileptics with diphenylhydantoin, phenobarbitone or primidone can lead to an effective folate deficiency

state. This action however, has never been satisfactorily related to the efficacy of the drug treatment and it seems likely that it is merely a side-effect of prolonged treatment with these particular anticonvulsant agents.

Undoubtedly, further research on the role of folic acid derivatives in cerebral function, both normal and abnormal, should be directed towards examining basic problems namely: which folic acid derivatives are present in brain and c.s.f.? is the folate activity in the c.s.f. derived from brain or plasma? which folic acid derivatives are transported across membranes, both at the tissue and cellular levels? and finally what are the effects of anticonvulsant drugs on the metabolism and transport of folic acid derivatives?

The potassium studies revealed that thiopentone, pentobarbitone and diazepam may reduce the c.s.f. potassium fluxes. This was interpreted as being due to an action of these drugs to decrease potassium exchange across the neuronal membrane. With diphenylhydantoin there was evidence for a slightly decreased potassium influx into the c.s.f. while paraldehyde increased c.s.f. potassium fluxes. There was therefore, no uniformity of action on potassium transport among these various agents, all of which have been used in the treatment of status epilepticus. This fact however does not disprove the hypothesis that an abnormally raised potassium level in the extracellular fluid is one of the factors contributing to epileptic discharges in some cases.

Direct testing of this hypothesis is made very difficult by virtue of the fact that sampling of brain extracellular fluid is impossible because of the smallness (several hundred angstroms across) of the intercellular spaces. Also in view of the smallness of the contribution which extracellular potassium makes to total brain potassium it is difficult to derive much useful information from total brain potassium levels. The most promising approach to this problem would seem to be to use glial membrane potential as a measure of extracellular potassium (review - Orkand, 1969). It is possible that this technique might allow direct analysis of the effect of anticonvulsant drugs on epileptic phenomena (w.r.t. extracellular potassium) at the neuronal level.

Of the three investigations perhaps the one with the greatest potential for application to the study of epilepsy in man is that concerning the role of amino acids. Preliminary results from the rats with the induced epileptic foci suggest that GABA, glutamate, glutamine and glycine may all be involved in epileptic processes. Similar investigations could be readily carried out on biopsy samples of epileptogenic tissue from patients undergoing operations for surgical removal of the focus.

However, for interpretation of simple amino acid levels in tissue it will be necessary to carry out further detailed studies in animals at the cellular level. Such studies should be directed towards elucidating the turnover of the amino acids, and investigating the possibility of separate and distinct pools of amino acids available for release as transmitter.

These investigations would be particularly relevant in view of the interest which is being shown in the use, as anticonvulsants, of drugs which specifically affect GABA metabolism.

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APPENDIX

Computer Records of Potassium Perfusion Experiments

```
%BEGIN
    %REALARRAY D(1:11)
    %INTEGER A,B
    %CYCLE B=1,1,23
    %CYCLE A=1,1,11
    READ (D(A))
    %REPEAT
    %BEGIN
        %REAL KAV
        %INTEGER M,K,L,N
        N=INT PT(D(3))
        %REALARRAY S(1:N,1:15)
        KAV=0
        %CYCLE M=1,1,N
        %CYCLE L=1,1,15
        S(M,L)=0
        %REPEAT
        %CYCLE K=1,1,8
        READ (S(M,K))
        %REPEAT
        %REPEAT
        %CYCLE M=1,1,N
        S(M,15)=S(M,8) / D(6)
        S(M,8) =D(5) / S(M,15) -D(5)
        S(M,2)=S(M,2)/D(10)
        S(M,3) =S(M,3) /D(10)
        S(M,4) =S(M,4) /D(11)
        S(M,5) =S(M,5) /D(11)
        S(M,2) =S(M,2) -S(M,4)
        S(M,3) = S(M,3) -S(M,5)
        S(M,4) = S(M,2) / S(M,3)
        S(M,2)=S(M,3)/(D(8)*S(M,4)+D(9))
        KAV=KAV+S(M,2)/D(4) %UNLESS M>D(4)
=> 1 %UNLESS M>D(4)
        S(M,9)=S(M,2)/KAV
        S(M,10)=(D(5)*KAV-S(M,2)*(D(5)+S(M,8)))
        S(M,11)=S(M,10)/((KAV/D(7)+S(M,2)/S(M,1))*0.5)
        S(M,12)=S(M,10)/((KAV+S(M,2))*0.5)
        S(M,13)=S(M,10)/(S(M,2)+0.37*(KAV-S(M,2)))
        S(M,14)=-(D(5)*(D(7)-S(M,1))+S(M,8)*(3.00-S(M,1))-S(M,11))
    11 %REPEAT
    NEWPAGE
    NEWLINES(3)
    SPACES(7)
    %PRINTTEXT'DATE=                DOG=                    PROCEDURE='
    NEWLINES(3)
    SPACES(6)
    %PRINTTEXT'                    INULIN      K       K 42        K 42'
    %PRINTTEXT'          K              K              K(1)'
    %PRINTTEXT'        K(2)'
    NEWLINE
    SPACES(6)
    %PRINTTEXT' SAMPLE  TIME     CLEARANCE   OUT      CHANNELS'
    %PRINTTEXT'   OUT/IN     EFFLUX     INFLUX        EFFLUX '
    %PRINTTEXT'    EFFLUX '
    NEWLINE
    SPACES(6)
    %PRINTTEXT'          MINS      ML/MIN      MEQ/L     RATIO       '
    %PRINTTEXT'RATIO      UEQ/MIN    UEQ/MIN       ML/MIN      ML/MIN'
    NEWLINES(2)
    %CYCLE M=1,1,N
    NEWLINE
    SPACES(8)
    PRINT(S(M,6),2,0)
    SPACES(3)
    PRINT(S(M,7),3,1)
```

```
67                      SPACES(3)
68                      PRINT(S(M,8),1,4)
69                      SPACES(4)
70                      PRINT(S(M,1),1,2)
71                      SPACES(4)
72                      PRINT(S(M,4),1,3)
73                      SPACES(5)
74                      PRINT(S(M,9),1,3)
75                      SPACES(5)
76                      PRINT(S(M,11),1,4)
77                      SPACES(4)
78                      PRINT(S(M,14),1,4)
79                      SPACES(6)
80                      PRINT(S(M,12),1,4)
81                      SPACES(4)
82                      PRINT(S(M,13),1,4)
83                      %REPEAT
84                      %END

85                  %REPEAT
86                  %ENDOFPROGRAM

   87 STATEMENTS COMPILED SUCCESSFULLY
CODE 5528 BYTES      GLAP 232+ 0 BYTES      DIAG TABLES 112 BYTES
LOADDATA 64 BYTES     TOTAL  5936 BYTES

OBJECT PROGRAM FILE COMPLETE.  PARAMETER FOR FILING := SPACE=(3438,2)
```

DATE- 8/12/70 DOG- A8 (INKY) PROCEDURE- CONTROL

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN
23	0.0	0.0000	3.15	0.522	0.000	0.0000	0.0000	0.0000	0.0000
24	0.0	0.0000	3.15	0.520	0.000	0.0000	0.0000	0.0000	0.0000
1	9.7	1.8583	3.28	0.951	0.002	1.8589	2.4182	0.5901	0.7963
2	17.1	1.7546	2.87	0.690	0.005	1.8227	1.5105	0.5789	0.7798
3	24.5	0.4353	2.81	0.561	0.152	1.0120	0.8272	0.3264	0.4036
4	31.9	0.2263	2.78	0.557	0.225	0.9101	0.7493	0.2960	0.3542
5	39.3	0.2325	2.79	0.539	0.212	0.9844	0.8339	0.3196	0.3846
6	46.7	0.1622	2.83	0.547	0.223	0.9940	0.8704	0.3221	0.3858
7	54.1	0.0968	2.84	0.531	0.284	0.8976	0.7891	0.2918	0.3413
8	61.5	0.1038	2.69	0.525	0.272	0.9098	0.7396	0.2994	0.3518
9	68.9	0.0911	2.72	0.522	0.280	0.9071	0.7526	0.2979	0.3490
10	76.3	0.0906	2.77	0.528	0.294	0.8731	0.7383	0.2858	0.3330
11	83.7	0.0827	2.73	0.531	0.315	0.8297	0.6814	0.2731	0.3159
12	91.1	0.0736	2.79	0.534	0.338	0.7932	0.6597	0.2600	0.2984
13	98.5	0.0641	2.76	0.522	0.349	0.7789	0.6465	0.2563	0.2931
14	105.9	0.0654	2.69	0.525	0.344	0.7828	0.6245	0.2594	0.2971
15	113.3	0.0672	2.78	0.523	0.352	0.7664	0.6426	0.2524	0.2883
16	120.7	0.0708	2.81	0.525	0.362	0.7438	0.6284	0.2437	0.2776
17	128.1	0.0672	2.78	0.521	0.369	0.7316	0.6458	0.2406	0.2734
18	135.5	0.0736	2.83	0.525	0.351	0.7641	0.6556	0.2497	0.2853
19	142.9	0.0750	2.90	0.529	0.371	0.7216	0.6391	0.2344	0.2662
21	157.7	0.0717	2.96	0.527	0.379	0.7145	0.6546	0.2308	0.2615
22	165.1	0.0722	2.96	0.525	0.359	0.7589	0.6990	0.2450	0.2793

DATE- 22 / 6 / 71 DOG- A7(JAFFA) PROCEDURE- CONTROL

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN
0	0.0	0.0000	2.65	0.712	0.000	0.0000	0.0000	0.0000	0.0000
0	0.0	0.0000	2.65	0.708	0.000	0.0000	0.0000	0.0000	0.0000
0	0.0	0.0000	2.66	0.707	0.000	0.0000	0.0000	0.0000	0.0000
11	61.9	0.0822	2.82	0.710	0.203	0.9892	1.1254	0.3695	0.4463
12	67.0	0.0576	2.85	0.711	0.284	0.8327	0.8840	0.3093	0.3618
13	72.1	0.0509	2.74	0.708	0.309	0.7827	0.7965	0.2931	0.3398
14	77.2	0.0401	2.75	0.712	0.323	0.7684	0.7883	0.2874	0.3315
15	82.2	0.0297	2.75	0.714	0.318	0.7925	0.8151	0.2965	0.3426
16	88.3	0.0195	2.73	0.716	0.305	0.8287	0.8475	0.3106	0.3605
17	92.4	0.0297	2.59	0.713	0.321	0.7742	0.7441	0.2938	0.3391
18	97.6	0.0250	2.75	0.710	0.336	0.7637	0.7875	0.2856	0.3279
19	102.6	0.0315	2.74	0.711	0.334	0.7581	0.7769	0.2837	0.3260
20	107.8	0.0322	2.72	0.712	0.338	0.7485	0.7605	0.2806	0.3221
21	112.8	0.0341	2.75	0.708	0.319	0.7843	0.8057	0.2933	0.3388
22	117.9	0.0356	2.70	0.697	0.325	0.7676	0.7719	0.2883	0.3324
23	123.0	0.0375	2.83	0.713	0.309	0.8039	0.8516	0.2988	0.3463
24	128.1	0.0401	2.76	0.710	0.294	0.8273	0.8606	0.3094	0.3605
25	133.2	0.0348	2.71	0.711	0.338	0.7442	0.7521	0.2793	0.3205
26	138.3	0.0348	2.80	0.713	0.314	0.7971	0.8352	0.2970	0.3437
27	143.4	0.0367	2.71	0.710	0.355	0.7095	0.7168	0.2662	0.3037
28	148.5	0.0409	2.73	0.709	0.353	0.7098	0.7227	0.2658	0.3036
29	153.6	0.0308	2.77	0.710	0.367	0.7015	0.7304	0.2616	0.2975
30	158.8	0.0401	2.73	0.707	0.334	0.7463	0.7594	0.2796	0.3213
31	163.9	0.0452	2.79	0.708	0.350	0.7122	0.7447	0.2652	0.3032
32	169.1	0.0452	2.73	0.710	0.365	0.6816	0.6934	0.2552	0.2903
33	174.2	0.0436	2.81	0.711	0.361	0.6959	0.7356	0.2586	0.2946
34	179.4	0.0409	2.79	0.709	0.369	0.6837	0.7172	0.2545	0.2892
35	183.6	0.0529	2.83	0.706	0.348	0.7089	0.7539	0.2631	0.3010

DATE-28/7/71 DOG- A7(JAFFA) PROCEDURE- CONTROL

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN
0	0.0	0.0000	2.64	0.981	0.000	0.0000	0.0000	0.0000	0.0000
0	0.0	0.0000	2.64	1.006	0.000	0.0000	0.0000	0.0000	0.0000
0	0.0	0.0000	2.64	0.981	0.000	0.0000	0.0000	0.0000	0.0000
1	2.3	0.9448	3.19	1.187	0.000	1.5832	1.9277	0.5997	0.8103
2	7.0	1.4751	3.00	0.975	0.003	1.5558	1.9638	0.5891	0.7947
3	11.4	1.1286	2.95	0.981	0.014	1.4561	1.4927	0.5507	0.7369
4	15.9	0.5547	2.95	0.977	0.055	1.2719	1.3372	0.4791	0.6245
5	21.0	0.5264	2.95	0.983	0.091	1.0976	1.1643	0.4121	0.5261
6	25.8	0.3383	2.87	0.982	0.112	1.0928	1.1178	0.4106	0.5181
7	30.4	0.2484	2.92	0.982	0.133	1.0700	1.1342	0.4008	0.5003
8	35.2	0.1967	2.91	0.981	0.149	1.0520	1.1153	0.3937	0.4876
9	40.2	0.1552	2.98	0.979	0.145	1.0934	1.1923	0.4082	0.5064
10	45.2	0.1412	2.97	0.982	0.159	1.0643	1.1590	0.3970	0.4894
11	50.2	0.1071	3.01	0.980	0.158	1.0918	1.2038	0.4066	0.5013
12	55.2	0.0881	2.98	0.981	0.177	1.0566	1.1568	0.3934	0.4808
13	60.2	0.0927	2.94	0.983	0.173	1.0613	1.1457	0.3960	0.4849
14	65.1	0.0856	2.94	0.982	0.171	1.0720	1.1569	0.4000	0.4903
15	70.0	0.0764	2.79	0.980	0.172	1.0687	1.0977	0.4016	0.4920
16	75.0	0.0891	2.92	0.981	0.178	1.0485	1.1253	0.3914	0.4780
17	79.9	0.0750	2.91	0.981	0.184	1.0463	1.1236	0.3906	0.4760
18	84.8	0.0891	2.85	0.980	0.179	1.0444	1.0940	0.3912	0.4778
19	89.5	0.0731	2.80	0.979	0.180	1.0510	1.0843	0.3946	0.4816
20	94.3	0.0731	2.75	0.981	0.188	1.0271	1.0418	0.3856	0.4700
21	99.1	0.0472	2.92	0.983	0.190	1.0535	1.1337	0.3929	0.4773
22	103.8	0.0432	2.96	0.983	0.207	1.0215	1.1158	0.3798	0.4581
23	108.5	0.0432	2.91	0.982	0.203	1.0278	1.1049	0.3832	0.4630
24	113.2	0.0542	2.88	0.982	0.202	1.0180	1.0835	0.3802	0.4596
25	117.8	0.0542	2.91	0.982	0.197	1.0305	1.1066	0.3844	0.4655
26	122.3	0.0542	2.90	0.981	0.198	1.0282	1.1008	0.3837	0.4646
27	126.6	0.0501	2.95	0.981	0.197	1.0366	1.1271	0.3859	0.4673
28	130.8	0.0542	2.98	0.980	0.207	1.0106	1.1115	0.3753	0.4525
29	135.0	0.0529	2.89	0.981	0.203	1.0170	1.0862	0.3796	0.4586
30	139.4	0.0521	2.91	0.980	0.205	1.0134	1.0897	0.3778	0.4560
31	144.2	0.0529	3.00	0.980	0.211	1.0052	1.1132	0.3728	0.4489
32	148.5	0.0542	2.99	0.982	0.211	1.0030	1.1074	0.3722	0.4481
33	153.7	0.0300	2.85	0.981	0.203	1.0348	1.0933	0.3871	0.4676
34	158.0	0.0513	2.91	0.982	0.212	0.9991	1.0755	0.3723	0.4481
35	162.4	0.0492	2.95	0.981	0.203	1.0235	1.1140	0.3808	0.4600
36	166.7	0.0645	2.96	0.982	0.203	1.0114	1.1049	0.3761	0.4545
37	171.1	0.0623	2.91	0.980	0.208	0.9975	1.0728	0.3718	0.4482
38	175.3	0.0663	2.89	0.983	0.215	0.9765	1.0442	0.3642	0.4378
39	179.6	0.0529	2.84	0.980	0.212	0.9936	1.0451	0.3717	0.4474
40	183.8	0.0563	2.83	0.981	0.220	0.9703	1.0177	0.3631	0.4354
41	188.1	0.0571	3.12	0.982	0.225	0.9732	1.1240	0.3582	0.4286
42	192.3	0.0610	3.11	0.982	0.242	0.9306	1.0783	0.3421	0.4066
43	196.5	0.0593	3.11	0.981	0.237	0.9437	1.0912	0.3471	0.4133
44	200.7	0.0610	3.06	0.980	0.235	0.9455	1.0752	0.3488	0.4158

DATE= 29 / 7 / 71 DOG= A10(DOUGAL) PROCEDURE= CONTROL

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN
0	0.0	0.0000	3.41	0.958	0.000	0.0000	0.0000	0.0000	0.0000
0	0.0	0.0000	3.41	0.960	0.000	0.0000	0.0000	0.0000	0.0000
0	0.0	0.0000	3.41	0.959	0.000	0.0000	0.0000	0.0000	0.0000
1	2.3	1.7134	2.98	0.968	0.006	1.9474	1.7841	0.5716	0.7691
2	6.9	0.4653	2.91	0.950	0.127	1.2031	1.0112	0.3596	0.4503
3	11.6	0.1823	2.93	0.948	0.245	0.9654	0.8087	0.2922	0.3470
4	16.7	0.1367	2.92	0.948	0.278	0.9191	0.7611	0.2794	0.3274
5	21.6	0.0989	2.92	0.947	0.307	0.8910	0.7360	0.2716	0.3150
6	26.7	0.0731	2.95	0.948	0.293	0.9706	0.8289	0.2947	0.3435
7	31.8	0.0750	2.96	0.950	0.325	0.8835	0.7455	0.2688	0.3098
8	36.9	0.0764	2.96	0.952	0.307	0.9292	0.7912	0.2822	0.3274
9	42.2	0.0641	2.93	0.952	0.331	0.8847	0.7362	0.2700	0.3106
10	47.4	0.0525	2.93	0.950	0.330	0.9040	0.7564	0.2759	0.3174
11	52.6	0.0708	2.78	0.949	0.325	0.8744	0.6599	0.2707	0.3120
12	57.8	0.1471	2.92	0.950	0.343	0.7132	0.6545	0.2181	0.2499
13	63.0	0.1267	2.89	0.952	0.360	0.7004	0.5305	0.2152	0.2452
14	68.3	0.0337	2.85	0.948	0.361	0.8547	0.6817	0.2637	0.3004
15	73.5	0.1027	2.91	0.950	0.371	0.7147	0.5555	0.2193	0.2490
16	78.6	0.0322	2.82	0.947	0.381	0.8099	0.6271	0.2512	0.2844
20	99.5	0.0534	2.82	0.953	0.379	0.7768	0.5982	0.2409	0.2729
21	104.7	0.0356	2.91	0.951	0.399	0.7713	0.6181	0.2373	0.2671
22	109.9	0.0333	2.82	0.951	0.429	0.7053	0.5223	0.2198	0.2453
23	115.1	0.0289	2.82	0.951	0.411	0.7518	0.5696	0.2339	0.2624
25	125.5	0.0289	2.81	0.955	0.397	0.7806	0.5951	0.2428	0.2735
26	130.6	0.0330	2.79	0.955	0.396	0.7719	0.5790	0.2406	0.2711
27	135.8	0.0322	2.81	0.956	0.391	0.7864	0.6003	0.2445	0.2758
28	141.0	0.0264	2.86	0.955	0.403	0.7759	0.6072	0.2401	0.2700
29	146.2	0.0272	2.86	0.954	0.402	0.7768	0.6080	0.2404	0.2704
30	151.4	0.0330	2.86	0.954	0.407	0.7559	0.5863	0.2340	0.2628
31	156.6	0.0219	2.88	0.957	0.411	0.7688	0.6072	0.2375	0.2664
32	161.8	0.0315	2.84	0.954	0.412	0.7463	0.5743	0.2317	0.2598
33	167.0	0.0272	2.92	0.954	0.412	0.7601	0.6110	0.2338	0.2622
34	172.4	0.0425	2.91	0.955	0.424	0.7058	0.5520	0.2176	0.2431
35	177.9	0.0460	2.91	0.954	0.445	0.6550	0.5009	0.2023	0.2247
36	183.2	0.0497	2.92	0.953	0.424	0.6916	0.5407	0.2130	0.2380
37	188.3	0.0488	2.94	0.955	0.430	0.6822	0.5383	0.2097	0.2339
38	193.6	0.0525	2.84	0.956	0.429	0.6688	0.4894	0.2080	0.2320
39	198.8	0.0571	2.99	0.956	0.429	0.6726	0.5460	0.2056	0.2294

DATE=1/4/69 DOG- A2(ELMO) PROCEDURE- THIOPENTONE — 113min

EXPT. 1

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
28	0.0	0.0000	2.67	0.361	0.000	0.0000	0.0000	0.0000	0.0000	
29	0.0	0.0000	2.87	0.360	0.000	0.0000	0.0000	0.0000	0.0000	
30	0.0	0.0000	2.87	0.359	0.000	0.0000	0.0000	0.0000	0.0000	
2	11.6	0.4958	2.86	0.374	0.172	0.7964	0.7240	0.2776	0.3400	
3	19.9	0.2545	2.84	0.358	0.229	0.8071	0.7574	0.2818	0.3367	
4	27.2	0.1680	2.84	0.371	0.277	0.7651	0.7293	0.2672	0.3134	
5	35.0	0.1392	2.78	0.359	0.290	0.7616	0.7040	0.2673	0.3119	
6	42.8	0.1412	2.83	0.367	0.297	0.7458	0.7098	0.2607	0.3035	
7	50.6	0.1304	2.83	0.351	0.290	0.7768	0.7427	0.2715	0.3169	
8	58.1	0.1196	2.80	0.362	0.301	0.7626	0.7177	0.2673	0.3107	
9	65.6	0.1167	2.78	0.358	0.310	0.7424	0.6897	0.2607	0.3020	
10	73.1	0.0699	2.85	0.357	0.401	0.6199	0.6034	0.2164	0.2435	
11	78.9	0.0764	2.78	0.353	0.379	0.6486	0.6048	0.2280	0.2582	
12	84.5	0.0764	2.80	0.364	0.393	0.6214	0.5851	0.2180	0.2459	
13	91.8	0.0750	2.80	0.362	0.393	0.6240	0.5880	0.2189	0.2469	Control
14	99.3	0.0623	2.83	0.368	0.418	0.5990	0.5764	0.2096	0.2346	
15	106.8	0.0610	2.80	0.354	0.408	0.6185	0.5853	0.2171	0.2437	
16	114.8	0.0632	2.79	0.357	0.417	0.5959	0.5586	0.2094	0.2344	
17	123.3	0.0356	2.81	0.359	0.533	0.4509	0.4262	0.1583	0.1719	
18	131.8	0.0401	2.79	0.354	0.547	0.4185	0.3951	0.1473	0.1594	
19	140.1	0.0356	2.80	0.362	0.568	0.3964	0.3682	0.1394	0.1501	
20	148.4	0.0460	2.78	0.368	0.569	0.3722	0.3351	0.1312	0.1413	
21	156.3	0.0425	2.72	0.357	0.548	0.4086	0.3517	0.1452	0.1571	
22	164.0	0.0425	2.81	0.358	0.549	0.4114	0.3854	0.1444	0.1563	Treatment
23	173.2	0.0405	2.81	0.359	0.549	0.4164	0.3907	0.1462	0.1582	
24	182.1	0.0394	2.90	0.366	0.607	0.3376	0.3427	0.1172	0.1251	
25	190.2	0.0390	2.81	0.361	0.612	0.3266	0.3312	0.1147	0.1224	
26	198.4	0.0348	2.81	0.362	0.585	0.3743	0.3497	0.1314	0.1410	

DATE- 8/4/69 DOG- A2(ELMO) PROCEDURE- THIOPENTONE — 97min

EXPT. 2

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
30	0.0	0.0000	2.88	0.359	0.000	0.0000	0.0000	0.0000	0.0000	
29	0.0	0.0000	2.88	0.359	0.000	0.0000	0.0000	0.0000	0.0000	
28	0.0	0.0000	2.88	0.360	0.000	0.0000	0.0000	0.0000	0.0000	
1	3.0	1.1151	2.81	0.370	0.024	1.4945	1.2617	0.5192	0.6903	
2	7.0	0.2425	2.87	0.357	0.097	1.2968	1.2523	0.4504	0.5730	
3	13.0	0.1155	2.85	0.357	0.126	1.2561	1.2398	0.4401	0.5515	
4	19.0	0.0713	2.87	0.358	0.345	0.7359	0.7237	0.2558	0.2929	
5	27.0	0.0517	2.78	0.360	0.380	0.6870	0.6457	0.2409	0.2728	
6	34.0	0.0272	2.79	0.362	0.421	0.6523	0.6196	0.2287	0.2558	
7	42.0	0.0222	2.87	0.359	0.428	0.6538	0.6479	0.2272	0.2537	
8	49.0	0.0319	2.78	0.360	0.410	0.6637	0.6267	0.2328	0.2613	
9	57.0	0.0538	2.79	0.362	0.406	0.6342	0.5959	0.2223	0.2497	
10	65.0	0.0436	2.77	0.368	0.417	0.6302	0.5871	0.2214	0.2479	
11	72.0	0.0417	2.76	0.363	0.426	0.6151	0.5691	0.2164	0.2416	
12	80.0	0.0233	2.79	0.373	0.425	0.6515	0.6196	0.2284	0.2552	Control
13	87.0	0.0261	2.76	0.359	0.434	0.6288	0.5865	0.2212	0.2465	
14	95.0	0.0297	2.56	0.364	0.413	0.6524	0.5763	0.2320	0.2601	
15	103.0	0.0468	2.83	0.359	0.405	0.6516	0.6286	0.2274	0.2556	
16	110.0	0.0398	2.77	0.361	0.425	0.6225	0.5804	0.2187	0.2444	
17	118.0	0.0304	2.79	0.359	0.431	0.6285	0.5951	0.2204	0.2458	
18	127.0	0.0275	2.73	0.365	0.441	0.6119	0.5595	0.2160	0.2403	
19	135.0	0.0247	2.71	0.357	0.447	0.6043	0.5462	0.2139	0.2375	
20	143.0	0.0279	2.74	0.361	0.467	0.5681	0.5189	0.2005	0.2214	
21	151.0	0.0257	2.77	0.361	0.473	0.5632	0.5243	0.1980	0.2183	Treatment
22	159.0	0.0250	2.76	0.363	0.482	0.5490	0.5070	0.1933	0.2126	
23	168.0	0.0268	2.73	0.363	0.476	0.5540	0.5017	0.1958	0.2157	
24	176.0	0.0344	2.83	0.361	0.461	0.5723	0.5515	0.1998	0.2211	
25	185.0	0.0378	2.84	0.359	0.457	0.5734	0.5554	0.2000	0.2215	

DATE- 28/10/70 DOG-A10(DOUGAL) PROCEDURE- THIOPENTONE — 126 min

EXPT. 3

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
28	0.0	0.0000	3.48	0.500	0.000	0.0000	0.0000	0.0000	0.0000	
27	0.0	0.0000	3.48	0.506	0.000	0.0000	0.0000	0.0000	0.0000	
26	0.0	0.0000	3.48	0.496	0.000	0.0000	0.0000	0.0000	0.0000	
1	9.7	0.9987	2.79	0.513	0.055	1.4860	1.0693	0.4325	0.5638	
2	17.1	0.3289	2.80	0.507	0.206	0.9423	0.6725	0.2820	0.3402	
3	24.5	0.1992	2.86	0.501	0.260	0.9006	0.6867	0.2704	0.3191	
4	31.9	0.1695	2.89	0.500	0.283	0.8660	0.6704	0.2601	0.3042	
5	39.3	0.1545	2.87	0.505	0.295	0.8510	0.6479	0.2564	0.2987	
6	46.7	0.1471	2.87	0.505	0.286	0.8901	0.6880	0.2679	0.3131	
7	54.1	0.1167	2.92	0.496	0.325	0.8247	0.6474	0.2481	0.2860	
8	61.5	0.1032	2.97	0.501	0.348	0.7899	0.6338	0.2371	0.2712	
9	68.9	0.1032	2.97	0.505	0.360	0.7575	0.6014	0.2276	0.2593	
10	76.3	0.0942	2.97	0.490	0.353	0.7909	0.6351	0.2375	0.2711	
11	83.7	0.0764	3.00	0.500	0.354	0.8234	0.6794	0.2465	0.2814	
12	91.1	0.0755	3.05	0.499	0.395	0.7269	0.6016	0.2172	0.2448	
13	98.5	0.0654	2.97	0.503	0.392	0.7480	0.5930	0.2253	0.2542	
14	105.9	0.0659	2.92	0.509	0.407	0.7071	0.5338	0.2145	0.2408	Control
15	113.3	0.0534	2.99	0.505	0.399	0.7552	0.6077	0.2272	0.2557	
16	120.7	0.0654	2.95	0.493	0.382	0.7696	0.6073	0.2321	0.2627	
17	128.1	0.0575	2.98	0.511	0.369	0.8183	0.6571	0.2458	0.2792	
18	135.5	0.0550	2.98	0.504	0.392	0.7672	0.6161	0.2309	0.2604	
19	142.9	0.0460	3.00	0.498	0.414	0.7361	0.5921	0.2214	0.2482	
20	150.3	0.0488	2.97	0.508	0.428	0.7016	0.5472	0.2120	0.2366	
21	157.7	0.0401	2.98	0.493	0.440	0.6902	0.5394	0.2085	0.2319	Treatment
22	165.1	0.0371	3.02	0.503	0.451	0.6779	0.5407	0.2040	0.2263	
23	172.5	0.0401	3.05	0.507	0.450	0.6755	0.5485	0.2026	0.2247	
24	179.9	0.0530	3.04	0.496	0.416	0.7115	0.5818	0.2132	0.2387	
25	187.3	0.0436	3.04	0.506	0.450	0.6675	0.5373	0.2004	0.2224	

DATE- 5 / 11 / 70 DOG- A1(SETH) PROCEDURE- THIOPENTONE — 155

EXPT. 4

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN
28	0.0	0.0000	2.75	0.504	0.000	0.0000	0.0000	0.0000	0.0000
29	0.0	0.0000	2.75	0.503	0.000	0.0000	0.0000	0.0000	0.0000
30	0.0	0.0000	2.75	0.505	0.000	0.0000	0.0000	0.0000	0.0000
1	9.7	0.5108	2.98	0.610	0.113	1.0384	1.0972	0.3747	0.4726
2	17.1	0.2000	2.96	0.547	0.287	0.6793	0.7343	0.2431	0.2840
3	24.5	0.1286	2.89	0.541	0.303	0.7262	0.7541	0.2611	0.3033
4	31.9	0.1000	2.89	0.527	0.322	0.7204	0.7514	0.2589	0.2987
5	39.3	0.0750	2.89	0.536	0.381	0.6341	0.6678	0.2275	0.2575
6	46.7	0.0750	2.97	0.542	0.322	0.7588	0.8225	0.2709	0.3126
7	54.1	0.0704	2.89	0.530	0.356	0.6899	0.7242	0.2477	0.2825
8	61.5	0.0448	2.91	0.541	0.400	0.6460	0.6900	0.2312	0.2602
9	68.9	0.0448	2.88	0.526	0.437	0.5797	0.6133	0.2079	0.2315
10	76.3	0.0448	2.89	0.524	0.416	0.6166	0.6537	0.2210	0.2476
11	83.7	0.0571	2.90	0.515	0.402	0.6220	0.6613	0.2228	0.2506
12	91.1	0.0448	2.90	0.507	0.416	0.6157	0.6572	0.2208	0.2473
13	98.5	0.0333	2.95	0.512	0.428	0.6189	0.6773	0.2205	0.2461
14	105.9	0.0251	2.92	0.511	0.400	0.6775	0.7264	0.2423	0.2726
15	113.3	0.0488	2.92	0.508	0.412	0.6201	0.6672	0.2217	0.2486
16	120.7	0.0488	2.89	0.507	0.407	0.6271	0.6638	0.2249	0.2526
17	128.1	0.0333	2.93	0.508	0.433	0.6080	0.6597	0.2170	0.2418
18	135.5	0.0344	2.87	0.511	0.470	0.5408	0.5723	0.1940	0.2141
19	142.9	0.0297	2.93	0.501	0.431	0.6190	0.6709	0.2209	0.2464
20	150.3	0.0371	2.90	0.510	0.443	0.5835	0.6248	0.2088	0.2321
21	157.7	0.0371	2.89	0.499	0.479	0.5234	0.5613	0.1873	0.2062
22	165.1	0.0448	2.85	0.501	0.482	0.5028	0.5261	0.1807	0.1988
23	172.5	0.0371	2.88	0.499	0.477	0.5254	0.5600	0.1883	0.2073
24	179.9	0.0448	2.82	0.505	0.487	0.4922	0.5051	0.1775	0.1950
25	187.3	0.0488	2.80	0.506	0.476	0.5020	0.5072	0.1815	0.2000
26	194.7	0.0448	2.89	0.507	0.461	0.5384	0.5754	0.1928	0.2132
27	202.1	0.0409	2.84	0.506	0.473	0.5237	0.5441	0.1885	0.2078

Control

Treatment

DATE= 25 / 11 / 70 DOG= A1 (SETH) PROCEDURE= THIOPENTONE — 133 min

EXPT. 5

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
30	0.0	0.0000	2.37	0.522	0.000	0.0000	0.0000	0.0000	0.0000	
31	0.0	0.0000	2.37	0.526	0.000	0.0000	0.0000	0.0000	0.0000	
32	0.0	0.0000	2.37	0.528	0.000	0.0000	0.0000	0.0000	0.0000	
1	3.5	0.6231	2.79	0.524	0.121	0.8101	0.8052	0.3362	0.4224	
2	11.3	0.3024	2.87	0.530	0.243	0.6063	0.7170	0.2471	0.2936	
3	18.8	0.2235	2.87	0.520	0.287	0.5741	0.6950	0.2328	0.2720	
4	26.3	0.1959	2.82	0.524	0.287	0.6013	0.7010	0.2447	0.2858	
5	33.8	0.1710	2.80	0.474	0.307	0.5850	0.6798	0.2379	0.2760	
6	41.3	0.1329	2.75	0.516	0.347	0.5470	0.6277	0.2226	0.2547	
7	48.8	0.1060	2.84	0.527	0.316	0.6447	0.7688	0.2612	0.3021	
8	56.3	0.1060	2.76	0.519	0.349	0.5771	0.6687	0.2346	0.2682	
9	63.8	0.0942	2.80	0.529	0.317	0.6533	0.7635	0.2655	0.3068	
10	71.3	0.0871	2.72	0.523	0.337	0.6213	0.7019	0.2536	0.2912	
12	86.3	0.0807	2.73	0.526	0.345	0.6157	0.7019	0.2510	0.2874	
13	93.8	0.0807	2.80	0.526	0.345	0.6193	0.7322	0.2510	0.2875	
14	101.3	0.0755	2.76	0.525	0.345	0.6236	0.7224	0.2536	0.2903	
15	108.8	0.0750	2.80	0.526	0.358	0.6026	0.7166	0.2440	0.2781	Control
16	116.3	0.0807	2.80	0.519	0.358	0.5954	0.7083	0.2411	0.2748	
17	123.8	0.0580	2.70	0.524	0.385	0.5749	0.6565	0.2343	0.2649	
18	131.3	0.0645	2.68	0.526	0.393	0.5516	0.6239	0.2251	0.2539	
19	138.8	0.0759	2.73	0.528	0.369	0.5789	0.6664	0.2356	0.2677	
20	146.3	0.0614	2.73	0.528	0.360	0.6132	0.7046	0.2497	0.2845	
21	153.8	0.0610	2.72	0.523	0.368	0.5999	0.6879	0.2444	0.2777	
22	161.3	0.0641	2.70	0.525	0.366	0.5975	0.6773	0.2439	0.2773	
23	168.8	0.0555	2.64	0.527	0.384	0.5762	0.6372	0.2362	0.2671	
24	176.3	0.0529	2.68	0.527	0.388	0.5748	0.6509	0.2347	0.2651	Treatment
25	183.8	0.0605	2.68	0.527	0.406	0.5361	0.6097	0.2187	0.2457	
26	191.3	0.0654	2.71	0.530	0.407	0.5292	0.6122	0.2152	0.2417	
27	198.6	0.0623	2.67	0.528	0.424	0.5041	0.5736	0.2056	0.2298	

DATE- 28/4/71 DOG- A1 (SETH) PROCEDURE- PENTOBARBITONE – 150 min

EXPT. 7

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
1	0.0	0.0000	2.82	0.653	0.000	0.0000	0.0000	0.0000	0.0000	
2	0.0	0.0000	2.82	0.658	0.000	0.0000	0.0000	0.0000	0.0000	
3	0.0	0.0000	2.82	0.646	0.000	0.0000	0.0000	0.0000	0.0000	
1	5.0	0.4059	2.93	0.691	0.152	0.9465	0.9511	0.3340	0.4129	
2	10.0	0.1237	3.03	0.686	0.330	0.6918	0.7585	0.2411	0.2775	
3	14.7	0.1011	2.94	0.695	0.348	0.6793	0.7092	0.2383	0.2727	
4	20.9	0.0841	2.97	0.683	0.306	0.7963	0.8388	0.2790	0.3237	
5	25.5	0.0769	3.00	0.693	0.361	0.6895	0.7435	0.2406	0.2740	
6	30.4	0.0645	2.92	0.676	0.367	0.6930	0.7179	0.2435	0.2769	
7	35.4	0.0645	2.95	0.687	0.379	0.6707	0.7064	0.2349	0.2661	
8	40.5	0.0559	2.92	0.666	0.369	0.7012	0.7267	0.2463	0.2799	
9	45.7	0.0382	2.96	0.657	0.422	0.6320	0.6724	0.2210	0.2470	
10	51.0	0.0341	2.95	0.667	0.416	0.6497	0.6870	0.2274	0.2547	
11	56.1	0.0432	2.95	0.654	0.394	0.6758	0.7126	0.2367	0.2668	
12	61.2	0.0440	2.95	0.656	0.397	0.6687	0.7055	0.2342	0.2638	
13	66.4	0.1862	2.95	0.675	0.375	0.4877	0.5174	0.1709	0.1938	
14	72.5	0.0755	2.96	0.667	0.322	0.7724	0.8376	0.2709	0.3126	
15	78.3	0.1249	3.00	0.664	0.143	1.1905	1.2445	0.4190	0.5205	
16	84.0	0.2425	2.97	0.704	0.077	1.3592	1.3959	0.4802	0.6181	
17	89.8	0.1739	3.10	0.694	0.151	1.1335	1.2349	0.3972	0.4915	
18	95.5	0.1000	2.99	0.659	0.238	0.9429	0.9929	0.3307	0.3937	
19	101.3	0.0645	2.94	0.661	0.269	0.9064	0.9385	0.3186	0.3748	
20	107.0	0.0353	3.00	0.664	0.365	0.7442	0.7982	0.2597	0.2954	
21	112.8	0.0275	2.88	0.648	0.371	0.7379	0.7526	0.2602	0.2954	
22	118.5	0.0363	2.89	0.667	0.317	0.8336	0.8506	0.2939	0.3397	
23	124.3	0.0356	2.95	0.664	0.337	0.7971	0.8343	0.2795	0.3209	Control
24	130.0	0.0243	2.85	0.653	0.360	0.7619	0.7672	0.2694	0.3070	
25	135.8	0.0212	2.83	0.673	0.367	0.7519	0.7513	0.2664	0.3028	
26	141.5	0.0229	2.87	0.687	0.391	0.7081	0.7201	0.2499	0.2820	
28	153.0	0.0185	2.73	0.657	0.478	0.5581	0.5651	0.2000	0.2202	
29	158.8	0.0229	2.71	0.665	0.488	0.5331	0.4935	0.1916	0.2104	
30	164.5	0.0145	2.80	0.666	0.549	0.4631	0.4543	0.1647	0.1782	
32	176.3	0.0145	2.78	0.675	0.566	0.4367	0.4215	0.1557	0.1677	
33	181.4	0.0236	2.79	0.655	0.581	0.3979	0.3840	0.1417	0.1522	
34	186.5	0.0308	2.80	0.651	0.586	0.3760	0.3639	0.1337	0.1434	
35	192.0	0.0282	2.80	0.649	0.611	0.3477	0.3361	0.1236	0.1319	Treatment
36	197.3	0.0289	2.80	0.647	0.616	0.3395	0.3277	0.1207	0.1287	
37	202.6	0.0282	2.78	0.657	0.611	0.3467	0.3285	0.1236	0.1319	
38	207.9	0.0375	2.81	0.666	0.609	0.3307	0.3206	0.1174	0.1253	
39	213.3	0.0341	2.80	0.663	0.600	0.3501	0.3373	0.1245	0.1332	

DATE- 4 / 8 / 71 DOG- A11 (QUEENIE) PROCEDURE- PENTOBARBITONE — 147 min

EXPT. 8

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
0	0.0	0.0000	2.06	0.895	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.06	0.891	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.06	0.888	0.000	0.0000	0.0000	0.0000	0.0000	
1	2.8	0.8029	2.82	0.921	0.068	0.8855	0.9690	0.4225	0.5467	
2	8.3	0.2137	2.82	0.946	0.184	0.7474	0.9369	0.3477	0.4236	
3	13.9	0.1458	2.71	0.903	0.251	0.6516	0.8043	0.3011	0.3566	
4	19.4	0.1310	2.75	0.889	0.270	0.6286	0.8029	0.2889	0.3396	
5	25.0	0.0974	2.86	0.935	0.257	0.6885	0.9148	0.3151	0.3724	
6	30.5	0.0563	2.75	0.930	0.279	0.6831	0.8760	0.3134	0.3672	
7	36.1	0.0589	2.80	0.941	0.280	0.6813	0.8916	0.3116	0.3650	
8	41.6	0.0497	2.75	0.895	0.322	0.6215	0.8160	0.2832	0.3268	
9	47.2	0.0261	2.78	0.891	0.343	0.6177	0.8279	0.2800	0.3208	
10	52.7	0.0261	2.64	0.893	0.344	0.6096	0.7742	0.2793	0.3198	
11	58.3	0.0212	2.55	0.894	0.434	0.4896	0.6270	0.2238	0.2494	
12	63.8	0.0135	2.82	0.888	0.378	0.5969	0.8225	0.2687	0.3052	
13	69.4	0.0165	2.93	0.894	0.334	0.6478	0.9076	0.2910	0.3344	
14	74.9	0.0352	2.78	0.899	0.370	0.5690	0.7773	0.2569	0.2918	
15	80.5	0.0250	2.75	0.897	0.382	0.5637	0.7645	0.2547	0.2882	
16	86.0	0.0250	2.75	0.894	0.388	0.5556	0.7563	0.2508	0.2833	
17	91.6	0.0261	2.72	0.893	0.385	0.5561	0.7468	0.2518	0.2846	
18	97.1	0.0261	2.78	0.887	0.366	0.5858	0.7960	0.2646	0.3010	
19	102.7	0.0250	2.58	0.887	0.379	0.5639	0.7419	0.2563	0.2903	
20	108.2	0.0250	2.59	0.887	0.393	0.5451	0.7263	0.2471	0.2787	Control
21	113.8	0.0202	2.71	0.891	0.410	0.5301	0.7192	0.2394	0.2686	
22	119.3	0.0165	2.57	0.885	0.396	0.5464	0.6923	0.2503	0.2821	
24	130.4	0.0165	2.57	0.890	0.420	0.5146	0.6605	0.2351	0.2630	
25	135.9	0.0191	2.51	0.890	0.432	0.4928	0.6184	0.2263	0.2523	
26	141.5	0.0165	2.60	0.884	0.439	0.4925	0.6479	0.2239	0.2492	
27	147.0	0.0222	2.64	0.886	0.395	0.5434	0.7394	0.2474	0.2788	
28	152.6	0.0155	2.64	0.893	0.418	0.5223	0.6907	0.2371	0.2654	
29	158.1	0.0145	2.49	0.893	0.422	0.5107	0.6323	0.2352	0.2630	
30	165.8	0.0185	2.60	0.890	0.433	0.4974	0.6520	0.2263	0.2523	
31	173.6	0.0145	2.56	0.895	0.453	0.4808	0.6559	0.2170	0.2406	
32	180.6	0.0145	2.74	0.906	0.455	0.4819	0.6822	0.2158	0.2391	
34	190.9	0.0119	2.65	0.887	0.435	0.5064	0.6793	0.2293	0.2554	Treatment
35	196.0	0.0155	2.55	0.888	0.446	0.4825	0.6225	0.2203	0.2447	
36	201.2	0.0341	2.55	0.889	0.454	0.4466	0.5782	0.2038	0.2258	
37	206.4	0.0282	2.46	0.887	0.452	0.4536	0.5584	0.2091	0.2318	
38	211.5	0.0272	2.39	0.889	0.442	0.4632	0.5456	0.2153	0.2394	
39	216.7	0.0289	2.42	0.892	0.457	0.4437	0.5349	0.2053	0.2273	

DATE- 21 / 4 / 69 DOG- A2(ELMO) PROCEDURE- DIAZEPAM — 116 min

EXPT. 9

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
26	0.0	0.0000	2.93	0.359	0.000	0.0000	0.0000	0.0000	0.0000	
27	0.0	0.0000	2.93	0.363	0.000	0.0000	0.0000	0.0000	0.0000	
28	0.0	0.0000	2.93	0.359	0.000	0.0000	0.0000	0.0000	0.0000	
1	1.7	1.6608	2.82	0.374	0.059	1.0217	0.6898	0.3495	0.4546	
2	7.4	0.3186	2.83	0.376	0.223	0.7699	0.6858	0.2645	0.3167	
3	15.9	0.2367	2.82	0.352	0.051	1.5185	1.4429	0.5192	0.6786	
4	25.5	0.1552	2.86	0.356	0.107	1.3258	1.2830	0.4536	0.5738	
5	34.5	0.1249	2.86	0.379	0.127	1.2755	1.2370	0.4365	0.5466	
6	42.6	0.1127	2.83	0.372	0.068	1.4873	1.4381	0.5088	0.6579	
7	50.9	0.0672	2.83	0.360	0.159	1.2145	1.1731	0.4165	0.5133	
8	61.8	0.0468	2.83	0.361	0.194	1.1352	1.0972	0.3897	0.4726	
9	72.6	0.0559	2.82	0.364	0.449	0.5597	0.5167	0.1933	0.2145	
10	79.3	0.0545	2.87	0.363	0.439	0.5661	0.5397	0.1944	0.2164	
11	88.1	0.0521	2.88	0.367	0.443	0.5820	0.5608	0.1997	0.2220	
12	96.9	0.0567	2.88	0.362	0.434	0.5896	0.5678	0.2023	0.2254	Control
13	104.1	0.0521	2.87	0.368	0.450	0.5679	0.5431	0.1951	0.2164	
14	111.8	0.0584	2.82	0.363	0.451	0.5524	0.5089	0.1908	0.2117	
15	120.0	0.0532	2.87	0.362	0.434	0.5786	0.5524	0.1987	0.2215	
16	128.3	0.0509	2.83	0.361	0.476	0.5228	0.4841	0.1805	0.1988	
17	136.4	0.0448	2.79	0.365	0.517	0.4622	0.4108	0.1604	0.1749	
18	144.5	0.0480	2.80	0.371	0.512	0.4651	0.4165	0.1612	0.1760	
19	154.1	0.0436	2.82	0.367	0.525	0.4525	0.4117	0.1565	0.1703	Treatment
20	164.9	0.0401	2.88	0.362	0.524	0.4647	0.4449	0.1596	0.1736	
21	175.3	0.0468	2.80	0.365	0.501	0.4856	0.4372	0.1683	0.1842	
22	185.6	0.0472	2.87	0.366	0.516	0.4635	0.4394	0.1593	0.1737	
23	195.9	0.0534	2.90	0.362	0.462	0.5459	0.5315	0.1869	0.2067	

DATE- 9 / 3 / 71 DOG-A7(JAFFA) PROCEDURE- DIAZEPAM — 124 min

EXPT. 10

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
40	0.0	0.0000	3.57	0.600	0.000	0.0000	0.0000	0.0000	0.0000	
41	0.0	0.0000	3.57	0.610	0.000	0.0000	0.0000	0.0000	0.0000	
42	0.0	0.0000	3.57	0.593	0.000	0.0000	0.0000	0.0000	0.0000	
1	6.3	3.9254	2.98	0.519	0.808	1.8707	1.6152	0.5249	0.7052	
2	11.6	1.2306	3.07	0.584	0.090	1.0514	0.9876	0.2985	0.3813	
3	16.5	0.6740	3.08	0.598	0.165	0.8374	0.7443	0.2398	0.2948	
4	22.3	0.5264	3.10	0.615	0.205	0.7525	0.6642	0.2162	0.2610	
5	27.4	0.4692	3.13	0.611	0.215	0.7728	0.7018	0.2219	0.2667	
6	32.5	0.4092	3.15	0.590	0.255	0.6607	0.5981	0.1901	0.2248	
7	37.8	0.3682	3.15	0.582	0.273	0.6427	0.5720	0.1852	0.2175	
8	43.2	0.3410	3.14	0.597	0.278	0.6607	0.5795	0.1906	0.2234	
9	48.3	0.3276	3.10	0.603	0.286	0.6473	0.5390	0.1874	0.2190	
10	53.4	0.3263	3.19	0.604	0.259	0.7625	0.7105	0.2188	0.2584	
11	58.6	0.3173	3.06	0.583	0.280	0.6826	0.5486	0.1982	0.2321	
12	64.2	0.3024	3.09	0.611	0.285	0.6900	0.5732	0.1999	0.2338	
13	69.5	0.2814	3.14	0.606	0.315	0.6157	0.5261	0.1781	0.2061	
14	75.1	0.2803	3.12	0.589	0.311	0.6303	0.5289	0.1826	0.2115	
15	80.4	0.2814	3.14	0.611	0.300	0.6673	0.5777	0.1928	0.2242	
16	86.1	0.2714	3.18	0.597	0.306	0.6642	0.5960	0.1914	0.2221	
17	92.3	0.2660	3.19	0.596	0.312	0.6517	0.5882	0.1877	0.2173	
18	98.1	0.2703	3.18	0.595	0.299	0.6912	0.6229	0.1991	0.2316	
19	103.3	0.2769	3.05	0.585	0.305	0.6537	0.5116	0.1904	0.2210	
20	108.2	0.2650	3.18	0.597	0.319	0.6309	0.5616	0.1820	0.2102	Control
21	113.0	0.2618	3.15	0.519	0.325	0.6127	0.5260	0.1772	0.2043	
22	117.6	0.2597	3.14	0.599	0.347	0.5404	0.4478	0.1567	0.1793	
23	124.4	0.2597	3.10	0.602	0.319	0.6349	0.5199	0.1844	0.2130	
24	130.3	0.2650	3.10	0.598	0.323	0.6108	0.4963	0.1774	0.2046	
25	135.4	0.2464	3.09	0.595	0.307	0.6962	0.5743	0.2021	0.2344	
26	140.5	0.2386	3.14	0.594	0.319	0.6719	0.5763	0.1944	0.2246	
27	145.2	0.2515	3.09	0.586	0.336	0.5892	0.4678	0.1715	0.1969	
28	150.4	0.2415	3.19	0.590	0.340	0.5995	0.5314	0.1730	0.1984	
29	155.1	0.2525	3.10	0.595	0.335	0.5920	0.4763	0.1721	0.1977	
30	159.3	0.2445	3.13	0.603	0.340	0.5898	0.4896	0.1711	0.1962	
31	164.3	0.2464	3.12	0.602	0.336	0.5991	0.4937	0.1739	0.1997	Treatment
32	169.3	0.2535	3.18	0.597	0.354	0.5323	0.4609	0.1539	0.1757	
33	174.2	0.2435	3.19	0.596	0.346	0.5747	0.5069	0.1659	0.1899	
34	179.3	0.2415	3.21	0.583	0.351	0.5635	0.5062	0.1624	0.1856	
35	184.5	0.2455	3.21	0.584	0.356	0.5420	0.4856	0.1563	0.1783	
36	189.6	0.2464	3.18	0.599	0.365	0.5096	0.4370	0.1474	0.1677	
37	195.1	0.2474	3.25	0.600	0.363	0.5185	0.4496	0.1489	0.1695	
38	200.2	0.2525	3.26	0.598	0.358	0.5254	0.4980	0.1509	0.1720	

DATE- 28 / 9 / 71 DOG-A9(MAGUA) PROCEDURE- DIAZEPAM — 142 min

EXPT. 11

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
0	0.0	0.0000	2.91	0.976	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.91	0.976	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.91	0.976	0.000	0.0000	0.0000	0.0000	0.0000	
17	58.4	0.0866	3.10	0.975	0.332	0.7608	0.8265	0.2574	0.2960	
18	62.0	0.0827	3.03	0.974	0.335	0.7573	0.7958	0.2577	0.2960	
19	65.6	0.0736	3.02	0.974	0.343	0.7508	0.7852	0.2556	0.2928	
20	69.2	0.0632	3.04	0.975	0.361	0.7308	0.7723	0.2483	0.2828	
21	72.9	0.0468	2.67	0.973	0.396	0.6607	0.6733	0.2329	0.2623	
22	76.4	0.0484	2.86	0.973	0.415	0.6355	0.6137	0.2195	0.2459	
23	80.1	0.0429	3.00	0.973	0.432	0.6238	0.6508	0.2124	0.2369	
24	83.7	0.0401	2.90	0.974	0.426	0.6327	0.6257	0.2177	0.2431	
25	87.3	0.0476	2.98	0.975	0.409	0.6569	0.6770	0.2242	0.2517	
26	90.9	0.0542	2.93	0.973	0.394	0.6714	0.6736	0.2303	0.2596	
27	94.5	0.0641	2.89	0.972	0.373	0.6943	0.6812	0.2390	0.2712	
28	98.1	0.0525	2.91	0.973	0.395	0.6703	0.6555	0.2303	0.2596	
29	101.8	0.0580	2.93	0.973	0.409	0.6347	0.6366	0.2177	0.2443	
30	105.4	0.0484	2.91	0.974	0.408	0.6521	0.6478	0.2241	0.2516	
31	109.0	0.0476	2.90	0.973	0.408	0.6528	0.6450	0.2245	0.2521	
32	112.6	0.0460	2.90	0.974	0.407	0.6586	0.6510	0.2265	0.2544	
33	116.2	0.0417	2.75	0.976	0.433	0.6067	0.5483	0.2122	0.2365	
34	119.8	0.0492	2.78	0.975	0.426	0.6091	0.5592	0.2122	0.2370	
35	123.4	0.0432	2.94	0.974	0.434	0.6156	0.6220	0.2109	0.2350	
36	127.0	0.0417	2.93	0.974	0.430	0.6246	0.6277	0.2142	0.2390	
37	130.6	0.0429	2.92	0.973	0.440	0.6040	0.6036	0.2073	0.2307	Control
38	134.1	0.0534	2.88	0.971	0.432	0.5969	0.5815	0.2058	0.2294	
39	137.7	0.0525	2.83	0.973	0.428	0.6020	0.5691	0.2086	0.2329	
40	141.6	0.0534	2.91	0.973	0.431	0.6015	0.5967	0.2067	0.2306	
41	145.0	0.0484	2.81	0.975	0.434	0.5978	0.5586	0.2076	0.2314	
42	148.4	0.0492	2.94	0.975	0.438	0.5974	0.6035	0.2047	0.2278	
43	152.5	0.0401	2.90	0.973	0.428	0.6281	0.6211	0.2161	0.2411	
44	156.1	0.0409	2.87	0.976	0.443	0.5983	0.5810	0.2065	0.2295	
45	159.1	0.0425	2.88	0.974	0.452	0.5793	0.5652	0.1997	0.2214	
46	162.6	0.0436	2.87	0.973	0.456	0.5701	0.5524	0.1968	0.2179	
47	166.1	0.0371	2.80	0.974	0.457	0.5757	0.5353	0.2003	0.2218	
48	169.7	0.0371	2.85	0.974	0.463	0.5696	0.5461	0.1970	0.2179	Treatment
49	173.2	0.0363	2.89	0.975	0.475	0.5525	0.5425	0.1903	0.2097	
50	176.8	0.0394	2.95	0.976	0.477	0.5458	0.5558	0.1867	0.2057	
51	180.3	0.0363	2.93	0.973	0.473	0.5574	0.5608	0.1911	0.2107	
52	183.9	0.0409	2.79	0.975	0.482	0.5262	0.4816	0.1833	0.2017	
53	187.5	0.0348	2.91	0.975	0.491	0.5290	0.5259	0.1818	0.1995	

DATE- 4/11/71 DOG- A9 (MAGUA) PROCEDURE- DIAZEPAM — 124 min

EXPT. 12

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
0	0.0	0.0000	2.16	0.680	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.16	0.675	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.16	0.681	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.16	0.685	0.000	0.0000	0.0000	0.0000	0.0000	
21	73.7	0.0717	2.63	0.691	0.370	0.5383	0.6527	0.2372	0.2694	
22	77.3	0.0425	2.64	0.690	0.393	0.5412	0.6699	0.2377	0.2681	
23	81.3	0.0452	2.55	0.689	0.404	0.5173	0.6140	0.2290	0.2574	
24	84.9	0.0275	2.55	0.694	0.426	0.5097	0.6143	0.2252	0.2515	
25	88.5	0.0236	2.57	0.681	0.442	0.4940	0.6009	0.2175	0.2418	
26	92.0	0.0212	2.60	0.684	0.428	0.5178	0.6413	0.2275	0.2540	
27	95.6	0.0261	2.62	0.692	0.424	0.5172	0.6453	0.2269	0.2536	
28	99.2	0.0161	2.63	0.683	0.431	0.5225	0.6575	0.2289	0.2553	Control
29	102.8	0.0161	2.64	0.681	0.437	0.5145	0.6527	0.2250	0.2505	
30	106.4	0.0138	2.59	0.689	0.473	0.4697	0.5931	0.2059	0.2270	
34	120.6	0.0102	2.65	0.682	0.471	0.4798	0.6232	0.2089	0.2305	
35	124.3	0.0161	2.65	0.689	0.459	0.4865	0.6279	0.2121	0.2347	
36	127.9	0.0161	2.69	0.689	0.438	0.5160	0.6700	0.2245	0.2499	
38	135.0	0.0112	2.62	0.684	0.472	0.4755	0.6093	0.2078	0.2291	
39	138.7	0.0102	2.60	0.685	0.469	0.4800	0.6079	0.2102	0.2320	
41	145.3	0.0148	2.70	0.684	0.467	0.4805	0.6381	0.2083	0.2300	
44	155.9	0.0212	2.68	0.684	0.484	0.4487	0.5979	0.1946	0.2139	
45	159.5	0.0185	2.68	0.682	0.506	0.4255	0.5756	0.1842	0.2013	
46	163.5	0.0250	2.62	0.681	0.516	0.4011	0.5295	0.1746	0.1904	Treatment
47	167.1	0.0161	2.67	0.685	0.507	0.4281	0.5757	0.1854	0.2027	
48	170.7	0.0452	2.69	0.684	0.507	0.3835	0.5284	0.1658	0.1812	
49	174.3	0.0452	2.66	0.682	0.503	0.3881	0.5228	0.1684	0.1843	
50	177.8	0.0439	2.71	0.680	0.515	0.3817	0.5349	0.1645	0.1795	

DATE- 12 / 5 / 71 DOG- A7 (JAFFA) PROCEDURE- DIPHENYLHYDANTOIN - 175 min

EXPT. 13

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
0	0.0	0.0000	3.21	0.662	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	3.21	0.667	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	3.21	0.663	0.000	0.0000	0.0000	0.0000	0.0000	
1	11.3	2.5037	3.09	0.681	0.000	1.9174	2.1067	0.5973	0.8070	
2	16.0	1.2789	3.00	0.678	0.032	1.5489	1.4859	0.4836	0.6395	
3	20.7	0.5333	2.85	0.666	0.108	1.2008	1.0128	0.3787	0.4789	
4	25.6	0.3579	2.89	0.666	0.147	1.1211	0.9857	0.3542	0.4391	
5	30.5	0.2587	2.89	0.667	0.178	1.0755	0.9511	0.3407	0.4162	
6	35.5	0.2017	2.90	0.679	0.219	0.9821	0.8689	0.3118	0.3741	
7	40.6	0.1886	2.93	0.665	0.221	0.9912	0.8940	0.3141	0.3765	
9	54.1	0.1559	2.93	0.683	0.224	1.0211	0.9262	0.3236	0.3876	
10	64.6	0.1323	2.93	0.700	0.247	0.9773	0.8840	0.3102	0.3680	
11	69.7	0.1310	2.88	0.699	0.221	1.0542	0.9395	0.3352	0.4019	
12	75.0	0.1399	2.88	0.678	0.226	1.0280	0.9123	0.3270	0.3912	
13	80.4	0.1412	2.90	0.678	0.249	0.9559	0.8488	0.3042	0.3605	
14	85.7	0.1310	2.96	0.676	0.240	1.0008	0.9205	0.3169	0.3769	
15	92.1	0.1286	2.94	0.664	0.249	0.9743	0.8856	0.3091	0.3663	
16	98.4	0.1196	2.96	0.670	0.267	0.9353	0.8556	0.2966	0.3491	
17	104.2	0.1178	2.92	0.687	0.268	0.9312	0.8347	0.2962	0.3484	
18	122.2	0.1127	2.98	0.674	0.265	0.9510	0.8798	0.3011	0.3546	
19	139.5	0.1149	3.00	0.680	0.255	0.9760	0.9150	0.3090	0.3653	
20	143.4	0.0937	2.96	0.705	0.242	1.0409	0.9621	0.3296	0.3918	Control
22	152.4	0.1399	2.95	0.702	0.150	1.2907	1.2057	0.4067	0.5034	
23	157.6	0.1360	2.96	0.687	0.205	1.1063	1.0258	0.3496	0.4220	
24	162.9	0.1208	2.92	0.680	0.226	1.0538	0.9572	0.3343	0.4000	
25	168.1	0.1167	2.95	0.681	0.246	1.0010	0.9171	0.3172	0.3765	
26	173.3	0.1110	2.96	0.677	0.267	0.9472	0.8677	0.3003	0.3535	
27	178.5	0.1104	2.96	0.664	0.283	0.9028	0.8226	0.2862	0.3348	
28	183.8	0.1021	2.94	0.671	0.305	0.8535	0.7664	0.2716	0.3152	
29	189.0	0.1016	2.93	0.673	0.286	0.9037	0.8126	0.2875	0.3360	
30	194.3	0.1065	2.96	0.664	0.266	0.9563	0.8770	0.3032	0.3570	
31	200.0	0.1016	2.93	0.668	0.279	0.9231	0.8320	0.2936	0.3439	
32	205.2	0.1000	2.95	0.667	0.283	0.9167	0.8337	0.2911	0.3406	
33	210.1	0.0881	2.95	0.664	0.270	0.9699	0.8875	0.3078	0.3619	Treatment
34	215.4	0.0932	2.96	0.662	0.272	0.9578	0.8791	0.3038	0.3569	
35	220.7	0.0953	2.93	0.664	0.295	0.8900	0.7993	0.2833	0.3300	
36	225.9	0.0984	2.90	0.667	0.313	0.8350	0.7322	0.2668	0.3087	
37	231.0	0.0984	2.89	0.660	0.340	0.7674	0.6605	0.2458	0.2819	
38	236.2	0.0979	2.93	0.663	0.337	0.7786	0.6877	0.2484	0.2852	

DATE- 25 / 5 / 71 DOG- A7(JAFFA) PROCEDURE- DIPHENYLHYDANTOIN - 148 min

EXPT. 14

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
0	0.0	0.0000	2.87	0.670	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.87	0.682	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.87	0.673	0.000	0.0000	0.0000	0.0000	0.0000	
1	11.3	4.6180	3.17	0.766	0.001	1.6987	2.5738	0.5918	0.7994	
2	16.0	1.2707	3.12	0.693	0.061	1.1080	1.3355	0.3843	0.4991	
3	20.7	0.6804	3.08	0.685	0.108	1.0144	1.1319	0.3511	0.4442	
4	25.6	0.4833	2.99	0.690	0.138	0.9738	1.0450	0.3377	0.4205	
5	30.4	0.3834	2.93	0.681	0.169	0.9076	0.8988	0.3153	0.3868	
6	35.4	0.2484	2.95	0.702	0.198	0.9210	0.9326	0.3195	0.3868	
7	40.5	0.2051	2.97	0.705	0.228	0.8697	0.8935	0.3011	0.3600	
8	45.6	0.2051	3.00	0.702	0.193	0.9821	1.0211	0.3398	0.4124	
9	53.4	0.1777	2.93	0.693	0.214	0.9373	0.9429	0.3254	0.3912	
10	61.2	0.1539	3.17	0.681	0.273	0.8112	0.9273	0.2769	0.3252	
11	69.0	0.1444	3.26	0.700	0.216	0.9842	1.1388	0.3356	0.4033	
12	76.7	0.1444	3.22	0.715	0.195	1.0428	1.1796	0.3569	0.4326	
13	82.6	0.1484	3.26	0.685	0.215	0.9838	1.1394	0.3355	0.4034	
14	87.9	0.1484	3.19	0.680	0.226	0.9471	1.0713	0.3239	0.3875	
15	93.2	0.1219	3.15	0.685	0.259	0.8866	0.9932	0.3031	0.3579	
16	98.6	0.1104	3.17	0.685	0.269	0.8746	0.9834	0.2986	0.3512	
17	104.0	0.1043	3.10	0.685	0.253	0.9204	0.9998	0.3159	0.3739	
18	109.4	0.1000	3.15	0.683	0.256	0.9194	1.0184	0.3145	0.3718	
19	114.8	0.0942	3.14	0.675	0.247	0.9488	1.0429	0.3249	0.3855	Control
20	120.9	0.0817	3.13	0.689	0.254	0.9464	1.0351	0.3242	0.3836	
21	129.3	0.0282	3.25	0.687	0.257	1.0097	1.1307	0.3434	0.4058	
22	136.8	0.0911	3.20	0.679	0.234	0.9902	1.1074	0.3383	0.4034	
23	142.6	0.0856	3.13	0.679	0.255	0.9373	1.0265	0.3211	0.3796	
24	147.8	0.0802	3.04	0.684	0.285	0.8664	0.9206	0.2981	0.3485	
25	152.5	0.0497	3.06	0.674	0.321	0.8285	0.8885	0.2843	0.3282	
26	157.7	0.0576	3.20	0.675	0.298	0.8765	0.9371	0.2982	0.3470	
27	162.8	0.0517	3.19	0.675	0.293	0.8955	1.0014	0.3049	0.3555	
28	168.0	0.0576	3.09	0.682	0.312	0.8383	0.9095	0.2871	0.3325	
29	173.2	0.0576	3.18	0.677	0.330	0.8057	0.9091	0.2739	0.3153	
30	178.4	0.0529	3.13	0.668	0.314	0.8430	0.9279	0.2879	0.3331	Treatment
31	183.5	0.0460	3.20	0.678	0.302	0.8825	0.9907	0.3001	0.3487	
32	188.7	0.0529	3.16	0.681	0.296	0.8846	0.9800	0.3017	0.3514	
33	193.9	0.0555	3.18	0.672	0.284	0.9105	1.0135	0.3104	0.3631	
34	199.1	0.0660	3.15	0.675	0.289	0.8831	0.9768	0.3016	0.3520	
35	204.3	0.0786	3.11	0.680	0.303	0.8311	0.9118	0.2844	0.3303	

DATE- 29 / 9 / 71 DOG-A10(DOUGAL) PROCEDURE-DIPHENYLHYDANTOIN - 142 min

EXPT. 15

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
53	0.0	0.0000	2.73	0.973	0.000	0.0000	0.0000	0.0000	0.0000	
54	0.0	0.0000	2.73	0.976	0.000	0.0000	0.0000	0.0000	0.0000	
55	0.0	0.0000	2.73	0.974	0.000	0.0000	0.0000	0.0000	0.0000	
1	6.0	1.6868	3.00	0.963	0.008	1.5397	1.6247	0.5636	0.7574	
2	9.4	0.5646	2.88	0.979	0.138	0.8708	0.8481	0.3170	0.3946	
3	12.9	0.1854	2.76	0.972	0.268	0.7321	0.6966	0.2676	0.3148	
4	16.5	0.1545	2.72	0.975	0.255	0.8008	0.7546	0.2936	0.3472	
5	20.1	0.1317	2.69	0.974	0.280	0.7611	0.7083	0.2797	0.3276	
6	23.7	0.0727	2.82	0.976	0.341	0.7093	0.7232	0.2577	0.2954	
7	27.4	0.1049	2.83	0.971	0.343	0.6611	0.6733	0.2400	0.2749	
8	31.0	0.1431	2.76	0.973	0.353	0.5805	0.5551	0.2120	0.2421	
9	34.6	0.0851	2.73	1.013	0.362	0.6430	0.6200	0.2355	0.2682	
10	38.2	0.0901	2.81	0.973	0.340	0.6868	0.6937	0.2498	0.2865	
11	41.9	0.0963	2.81	0.973	0.324	0.7132	0.7189	0.2594	0.2991	
12	45.6	0.0851	2.80	0.976	0.341	0.6902	0.6942	0.2512	0.2880	
13	50.2	0.0736	2.94	0.975	0.359	0.6802	0.7388	0.2445	0.2787	
14	54.9	0.0681	2.90	0.974	0.351	0.7007	0.7449	0.2528	0.2888	
15	58.9	0.0717	2.86	0.975	0.330	0.7354	0.7544	0.2664	0.3065	
16	62.7	0.0659	2.85	0.972	0.350	0.7039	0.7300	0.2550	0.2915	
17	66.2	0.0559	2.85	0.974	0.359	0.6992	0.7268	0.2533	0.2886	
18	69.8	0.0610	2.82	0.974	0.345	0.7184	0.7344	0.2610	0.2988	
19	73.5	0.0484	2.80	0.973	0.362	0.7013	0.7126	0.2552	0.2906	
20	77.8	0.0525	2.81	0.976	0.372	0.6769	0.6909	0.2460	0.2793	
21	82.0	0.0645	2.84	0.974	0.336	0.7317	0.7544	0.2654	0.3047	
22	85.8	0.0559	2.84	0.975	0.369	0.6803	0.7044	0.2466	0.2802	
23	89.6	0.0492	2.85	0.975	0.372	0.6844	0.7130	0.2478	0.2813	
24	93.3	0.0492	2.81	0.973	0.376	0.6743	0.6989	0.2451	0.2778	
25	97.3	0.0529	2.82	0.974	0.382	0.6576	0.6751	0.2388	0.2701	
26	101.7	0.0517	2.83	0.974	0.376	0.6726	0.6938	0.2440	0.2766	
27	105.9	0.0501	2.84	0.974	0.344	0.7364	0.7614	0.2671	0.3059	
28	109.6	0.0501	2.84	0.973	0.351	0.7229	0.7479	0.2621	0.2996	Control
29	113.6	0.0468	2.86	0.975	0.334	0.7625	0.7950	0.2761	0.3173	
30	117.7	0.0444	2.83	0.974	0.343	0.7468	0.7692	0.2711	0.3106	
31	121.7	0.0444	2.90	0.977	0.356	0.7253	0.7719	0.2616	0.2985	
32	125.7	0.0509	2.87	0.977	0.344	0.7369	0.7723	0.2666	0.3053	
33	129.8	0.0534	2.85	0.972	0.362	0.6969	0.7249	0.2524	0.2874	
34	134.1	0.0444	2.82	0.974	0.381	0.6737	0.6927	0.2446	0.2769	
35	138.4	0.0567	2.88	0.973	0.367	0.6851	0.7233	0.2474	0.2813	
36	142.3	0.0534	2.87	0.975	0.374	0.6758	0.7109	0.2443	0.2771	
37	146.2	0.0525	2.84	0.975	0.375	0.6738	0.6984	0.2442	0.2770	
38	149.9	0.0452	2.86	0.974	0.398	0.6441	0.6768	0.2329	0.2623	
39	153.6	0.0425	2.83	0.973	0.413	0.6188	0.6416	0.2243	0.2515	
40	157.3	0.0417	2.85	0.974	0.380	0.6816	0.7113	0.2468	0.2794	
42	164.7	0.0436	2.80	0.973	0.379	0.6765	0.6887	0.2461	0.2787	
43	168.4	0.0444	2.76	0.973	0.421	0.5978	0.6961	0.2183	0.2441	
44	172.1	0.0452	2.82	0.969	0.384	0.6660	0.6848	0.2418	0.2734	
45	175.8	0.0452	2.80	0.973	0.410	0.6187	0.6307	0.2250	0.2525	Treatment
46	179.5	0.0341	2.76	0.975	0.447	0.5701	0.5709	0.2081	0.2311	
47	183.2	0.0304	2.82	0.975	0.409	0.6449	0.6564	0.2340	0.2627	
48	187.0	0.0363	2.80	0.974	0.418	0.6190	0.6328	0.2251	0.2520	
49	190.7	0.0386	2.76	0.973	0.400	0.6434	0.6431	0.2349	0.2644	
50	194.5	0.0444	2.79	0.975	0.373	0.6867	0.6954	0.2501	0.2838	

52	198.3	0.0452	2.80	0.975	0.376	0.6805	0.6924	0.2475	0.2807

expt. 15

DATE- 18 / 6 / 69 DOG- A5(HORATIO) PROCEDURE- PARALDEHYDE - 111 min

EXPT. 16

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
25	0.0	0.0000	3.13	0.293	0.000	0.0000	0.0000	0.0000	0.0000	
26	0.0	0.0000	3.13	0.289	0.000	0.0000	0.0000	0.0000	0.0000	
3	23.0	0.2814	3.04	0.383	0.282	0.6586	0.6431	0.2119	0.2479	
4	32.9	0.1310	2.97	0.305	0.315	0.7720	0.7201	0.2498	0.2890	
5	42.7	0.0861	2.98	0.291	0.328	0.8063	0.7596	0.2608	0.3003	
6	52.6	0.0989	2.95	0.305	0.354	0.7228	0.6638	0.2346	0.2678	
7	62.5	0.0722	2.96	0.297	0.403	0.6640	0.6101	0.2156	0.2426	
8	72.4	0.0601	2.96	0.299	0.429	0.6271	0.5737	0.2038	0.2275	
9	82.3	0.0601	2.98	0.299	0.422	0.6430	0.5966	0.2085	0.2332	
10	92.2	0.0641	2.93	0.294	0.441	0.5927	0.5282	0.1933	0.2150	Control
11	102.1	0.0708	2.92	0.283	0.393	0.6796	0.6110	0.2215	0.2499	
12	112.0	0.0708	2.89	0.296	0.407	0.6473	0.5675	0.2118	0.2378	
13	121.9	0.0764	2.98	0.315	0.414	0.6289	0.5824	0.2039	0.2285	
14	131.8	0.0555	2.98	0.312	0.398	0.6989	0.6528	0.2265	0.2550	
15	141.7	0.0529	2.93	0.297	0.434	0.6283	0.5646	0.2049	0.2283	
16	151.7	0.0563	2.96	0.296	0.416	0.6590	0.6057	0.2141	0.2398	
17	161.8	0.0468	2.92	0.300	0.413	0.6809	0.6142	0.2221	0.2490	
18	171.9	0.0480	2.98	0.294	0.395	0.7188	0.6729	0.2329	0.2625	
19	182.1	0.0845	2.91	0.292	0.366	0.7141	0.6405	0.2328	0.2647	Treatment
20	192.3	0.0681	2.88	0.288	0.413	0.6388	0.5556	0.2093	0.2346	
21	202.3	0.0764	2.85	0.287	0.413	0.6232	0.5278	0.2048	0.2297	
22	212.2	0.0330	2.98	0.296	0.431	0.6737	0.6280	0.2185	0.2437	
23	222.4	0.0333	2.94	0.290	0.428	0.6761	0.6171	0.2202	0.2458	
24	232.5	0.0195	2.94	0.297	0.418	0.7220	0.6638	0.2350	0.2632	

DATE- 30 / 9 / 71 DOG- A7 (JAFFA) PROCEDURE- PARALDEHYDE - 125 min

EXPT. 18

SAMPLE	TIME MINS	INULIN CLEARANCE ML/MIN	K OUT MEQ/L	K 42 CHANNELS RATIO	K 42 OUT/IN RATIO	K EFFLUX UEQ/MIN	K INFLUX UEQ/MIN	K(1) EFFLUX ML/MIN	K(2) EFFLUX ML/MIN	
0	0.0	0.0000	2.70	0.974	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.70	0.974	0.000	0.0000	0.0000	0.0000	0.0000	
0	0.0	0.0000	2.70	0.974	0.000	0.0000	0.0000	0.0000	0.0000	
8	29.3	0.1329	2.79	0.973	0.117	1.2104	1.2495	0.4468	0.5625	
9	32.6	0.1208	2.82	0.978	0.131	1.1763	1.1946	0.4335	0.5419	
10	35.9	0.1049	2.82	0.978	0.152	1.1235	1.1406	0.4138	0.5116	
11	39.3	0.0896	2.82	0.981	0.163	1.1053	1.1252	0.4069	0.5007	
12	42.5	0.0793	2.97	0.972	0.166	1.1109	1.1895	0.4061	0.4988	
13	46.0	0.0793	2.95	0.976	0.174	1.0891	1.1601	0.3983	0.4874	
14	49.6	0.0690	2.91	0.974	0.178	1.0848	1.1416	0.3974	0.4854	
15	53.1	0.0841	2.93	0.979	0.145	1.1638	1.2259	0.4267	0.5295	
16	56.7	0.0783	2.99	0.973	0.155	1.1439	1.2301	0.4182	0.5164	
17	60.3	0.0783	3.01	0.973	0.157	1.1389	1.2326	0.4159	0.5131	
18	63.8	0.0601	2.78	0.974	0.154	1.1497	1.1945	0.4242	0.5242	
19	67.4	0.0932	2.93	0.974	0.147	1.1524	1.2148	0.4225	0.5238	
20	70.9	0.0932	2.96	0.978	0.136	1.1842	1.2385	0.4340	0.5410	
21	74.5	0.0708	3.03	0.971	0.160	1.1377	1.2389	0.4151	0.5114	
22	78.1	0.0672	2.90	0.972	0.176	1.0909	1.1442	0.3999	0.4888	
23	81.7	0.0610	2.99	0.976	0.172	1.1118	1.1982	0.4059	0.4972	
24	85.3	0.0663	2.95	0.977	0.180	1.0864	1.1581	0.3972	0.4849	
25	88.9	0.0736	2.94	0.977	0.166	1.1151	1.1827	0.4082	0.5015	
26	92.5	0.0645	2.87	0.972	0.173	1.1009	1.1436	0.4042	0.4950	
27	96.0	0.0901	2.84	0.978	0.176	1.0711	1.0987	0.3938	0.4816	
28	99.6	0.0942	3.03	0.973	0.183	1.0567	1.1585	0.3847	0.4689	
29	103.1	0.0953	2.89	0.976	0.175	1.0722	1.1188	0.3932	0.4811	Control
30	106.7	0.0871	2.99	0.974	0.193	1.0349	1.1211	0.3773	0.4577	
31	110.3	0.0990	3.00	0.972	0.189	1.0892	1.1792	0.3970	0.4826	
32	113.8	0.0892	2.99	0.975	0.190	1.0781	1.1646	0.3931	0.4777	
33	117.4	0.0829	2.96	0.975	0.188	1.0851	1.1614	0.3963	0.4819	
34	120.9	0.0534	2.91	0.977	0.196	1.0550	1.1132	0.3861	0.4679	
35	124.5	0.0610	2.99	0.973	0.202	1.0360	1.1224	0.3774	0.4561	
36	128.0	0.0525	2.99	0.975	0.192	1.0695	1.1560	0.3899	0.4734	
37	131.6	0.0468	2.95	0.985	0.195	1.0655	1.1381	0.3892	0.4719	
38	135.2	0.0429	2.98	0.972	0.202	1.0514	1.1277	0.3836	0.4636	
39	138.8	0.0413	2.85	0.975	0.202	1.0465	1.0953	0.3842	0.4642	
40	142.3	0.0421	2.90	0.976	0.212	1.0260	1.0818	0.3754	0.4518	
41	145.9	0.0475	2.98	0.975	0.208	1.0355	1.1186	0.3773	0.4549	
42	149.5	0.0436	2.98	0.977	0.216	1.0165	1.1016	0.3709	0.4455	
43	153.0	0.0421	3.01	0.975	0.222	1.0100	1.1034	0.3671	0.4400	
44	156.6	0.0398	2.91	0.977	0.223	1.0041	1.0836	0.3670	0.4397	Treatment
45	160.2	0.0359	2.89	0.973	0.217	1.0185	1.0715	0.3728	0.4476	
46	163.7	0.0322	2.96	0.973	0.222	1.0156	1.0923	0.3701	0.4435	
47	167.3	0.0436	2.96	0.974	0.227	0.9923	1.0686	0.3616	0.4323	
48	170.9	0.0375	3.04	0.975	0.217	1.0262	1.1297	0.3725	0.4473	
49	174.4	0.0517	3.03	0.973	0.208	1.0336	1.1341	0.3756	0.4528	
50	178.0	0.0550	3.01	0.974	0.212	1.0202	1.1138	0.3711	0.4466	
51	181.6	0.0593	3.11	0.973	0.202	1.0438	1.1733	0.3780	0.4568	
52	185.1	0.0593	3.00	0.982	0.207	1.0263	1.1163	0.3736	0.4505	

STOPPED AT LINE 86

LACK OF EFFECT OF FOLIC-ACID ADMINISTRATION OF CEREBRAL METABOLISM

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LACK OF EFFECT OF FOLIC-ACID ADMINISTRATION ON CEREBRAL METABOLISM

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Summary The administration of folic acid, which has been reported to cause alterations in mood and seizure threshold, was found to be without effect on the concentration of homovanillic acid, 5-hydroxyindol-3-ylacetic acid, and even the folate activity in the cerebrospinal fluid of dogs. The findings are against the central toxic actions which have been proposed for folic acid.

INTRODUCTION

UNCONTROLLED trials reporting that folic acid produces " mental changes " in volunteers [1] and that when given concurrently with vitamin B_{12} it alleviates mental retardation in epileptic patients on drug therapy [2] have received much criticism.[3–7] There have also been several trials,[4,6,8–10] some of them carefully controlled, which have shown that pharmacological doses of folic acid do not appear to influence mood or behaviour in epileptic patients or volunteers. In spite of this, there is some recent support for the thesis [11] that folic-acid metabolism influences mental function, in that certain metabolic errors causing low folate concentration are associated with mental defect.[12,13] It is also claimed that " there is a significant association between a variety of psychiatric diagnoses in epileptic patients and disturbed folate metabolism " [14] and that " there was a definite, though diagnostically indeterminate, relationship between low serum folate and mental illness in epileptic patients ".[6]

Pteridine derivatives are the coenzymes for tyrosine hydroxylase [15] and tryptophan hydroxylase,[16] the rate-limiting steps in the synthesis of dopamine and of 5-hydroxytryptamine (5-H.T.), and alterations in these biogenic amines in brain have been found to be associated with mood changes. It has been suggested [1] that these now well-documented findings provide a

rational basis for the influence of the administration of folic acid on mood and behaviour. While it may be naive to consider that folate activity as measured by bacteriological assay may indicate the availability of pteridine coenzyme in a suitably reduced form, it nevertheless seemed worth while to examine more closely the relationship of these pathways in brain in the in-vivo situation.

METHODS

Cerebrospinal fluid (C.S.F.) was withdrawn from the lateral ventricle and cisterna magna of beagle dogs [17] twice weekly at approximately 10 A.M. These samples were assayed for folate,[18] 5-hydroxyindol-3-ylacetic acid (5-H.I.A.A.) (the acid metabolite of 5-H.T.), and homovanillic acid (H.V.A.) (the main acid metabolite of dopamine).[19] Samples of plasma which were obtained concurrently had portions subjected to ultrafiltration,[20] and folate activity was estimated [18] in both whole plasma and ultrafiltrate of plasma. Once a control baseline had been established for each dog, 2·5 mg. folic acid was administered orally at 9 A.M. and 5 P.M. each day throughout the next month, and further samples were taken throughout the treatment period. (This dose on a weight-for-weight basis is equivalent to a daily intake in man of 30 mg., twice the normal pharmacological dose used in most studies.) The dogs were fed a constant amount of a batch-prepared diet for three weeks prior to the start of the control period and throughout the entire experiment.

RESULTS

It has been demonstrated that there is a gradient

TABLE I—HOMOVANILLIC ACID (H.V.A.) AND 5-HYDROXYINDOLYLACETIC ACID (5-H.I.A.A.) BEFORE AND AFTER CHRONIC FOLIC-ACID ADMINISTRATION

Sample	Dog	5-H.I.A.A. (ng./ml.)*		H.V.A. (ng./ml.)*	
		Before	After	Before	After
C.S.F. from lateral ventricle	1	297±53 (9)	250±45 (7)	1768±215 (10)	1977±542 (7)
	2	297±43 (6)	159±111 (6)	1137±446 (7)	1019±477 (6)
	3	131±47 (5)	137±47 (5)	†279±223 (6)	1211±670 (5)
C.S.F. from cisterna magna	1	41±7 (7)	35±6 (5)	114±33 (9)	103±41 (6)
	2	19±6 (5)	20±5 (4)	36±8 (6)	39±18 (3)
	3	37±8 (4)	34±25 (2)	99±35 (5)	114±48 (2)

* Mean±standard deviation (number of samples in parentheses).

† Abnormally low initial values due to contamination with blood, control values taken three months after stopping folic-acid administration 1501±1009 (16).

in the concentrations of the acid metabolites, 5-H.I.A.A. and H.V.A., from lateral ventricle to cisterna magna,[21] and that this is due to a localised transport system for the removal of the metabolites in the region of the fourth ventricle.[22] The results (table I) confirm the presence of this gradient. The administration of folic acid did not alter the concentration of 5-H.I.A.A. or H.V.A. in C.S.F. from either the lateral ventricle or cisterna magna.

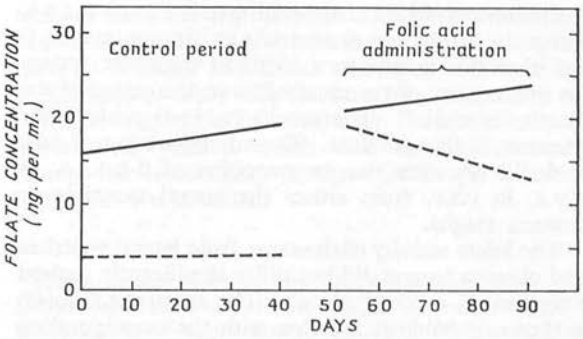
The folate activity of the C.S.F. from lateral ventricle and cisterna magna did not differ significantly (paired *t* test on 38 control values). The mean C.S.F.-folate is shown in table II together with the corresponding concentrations found in whole plasma and plasma ultrafiltrate. Control samples showed that, as in man,[23–27] the folate activity was considerably higher in C.S.F. than in plasma. However, in contrast to previous studies,[24,25,27] there was no significant correlation between plasma-folate activity and the comparable C.S.F. values, thus confirming the findings of a recent extensive study of 491 paired samples in man.[26] It would appear that, as in man,[28] much of the folate activity in dog plasma is protein-bound, since in the control period the plasma ultrafiltrate has only 43±3% of the folate activity of whole plasma. There was no significant correlation between folate activity in paired samples of C.S.F. and plasma ultrafiltrate.

Following the administration of folic acid, the folate activity in whole plasma showed a tenfold rise. The proportion of plasma-folate activity which was filterable decreased to 21±2%; thus there was only a

TABLE II—FOLATE ACTIVITY BEFORE AND AFTER CHRONIC FOLIC-ACID ADMINISTRATION

Sample	Dog	Folate (ng./ml.)*		Significance (*t* test)
		Before	After	
C.S.F.	1	20±4 (11)	22±2 (8)	..
	2	14±5 (9)	17±5 (8)	..
	3	18±4 (7)	29±4 (7)	..
Plasma ultrafiltrate ..	1	3·7±0·8 (8)	16±8 (3)	<0·01
	2	3·4±0·8 (6)	18±5 (6)	<0·001
	3	3·3±0·3 (7)	14±7 (5)	<0·01
Plasma	1	9·3±4·1 (8)	83±33 (3)	<0·001
	2	8·1±4·8 (6)	91±27 (6)	<0·001
	3	7·1±2·4 (7)	62±17 (5)	<0·001

* Mean±standard deviation (number of samples in parentheses).



Folate activity in plasma ultrafiltrate (interrupted line) and C.S.F. (continuous line) before and after folic-acid administration.

fivefold increase in folate activity in plasma ultrafiltrate. These changes were significant in all the dogs (table II). Despite the considerable increases in folate activity in plasma and its ultrafiltrate, there was only a small increase in C.S.F. folate, and in view of the slight positive regression of folate in C.S.F. during the control period (see accompanying figure), no change in C.S.F. folate activity can be attributed to the folic-acid supplements.

DISCUSSION

Alterations in cerebral metabolism of the biogenic amines are reflected in the concentrations of their acid metabolites in C.S.F.[29] It has been said [30] that administration of folic acid caused a significant lowering of H.V.A. levels which related to raised serum-folate concentrations. This is at variance with our results and those from a trial of patients with senile dementia,[27] where in both instances folic-acid administration caused no alterations in the concentrations of H.V.A. or 5-H.I.A.A. in C.S.F. Indeed, it seems that oral administration of folic acid, although increasing folate activity markedly in plasma and plasma ultrafiltrate, does not do so in C.S.F., and may therefore not be expected to alter folate activity in brain. The studies confirm the lack of effect of folic-acid administration on C.S.F. folate activity found recently in epileptics on anticonvulsant therapy [31] and in patients with senile dementia.[27]

The lack of increase of folate activity in C.S.F. may well be explained by the finding that an oral dose of

folic acid does not appear to cause a rise of 5-methyltetrahydrofolate in plasma,[32] and this is apparently one of the forms of folate that is rapidly transferred to C.S.F.[33] Indeed, it has been suggested[34] on the basis of in-vitro enzyme studies that a high plasma concentration of folic acid in its fully oxidised form will cause a pronounced inhibition of dihydrofolate reductase, thus impairing the conversion of dihydrofolate to tetrahydrofolate (the other form of folate rapidly transferred to C.S.F.[33]) and thus possibly even reducing the amount of methyltetrahydrofolate present in plasma. This suggestion agrees well with our present data and with studies in man of the absorption and metabolism of different forms of radioactively labelled folate as indicated by the correlations found between radioactivity and assay of folate activity with both *Lactobacillus casei* and *Streptococcus fæcalis*.[32,35]

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Effect of drugs used in status-epilepticus on the potassium fluxes of cerebrospinal fluid in the conscious dog

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Zuckerman & Glazer (1968) demonstrated that localized high concentrations of potassium in the region of the inferior horn of the lateral ventricle could cause convulsions originating in the hippocampal system.

The potassium concentration in the cerebrospinal fluid (C.S.F.) is maintained within narrow limits independent of large alterations in plasma concentrations (Kemeny, Boldizsar & Pethes, 1961). The mechanisms whereby this is achieved have been investigated and characterized by ventriculo-cisternal perfusion techniques (Heisey, Held & Pappenheimer, 1962; Cserr, 1965; Katzman, Graziani & Ginsberg, 1968; Bradbury & Stulcova, 1970). In the experiments described here, drugs useful in the treatment of status epilepticus were investigated for their effect on the potassium fluxes of C.S.F.

Ventriculo-cisternal perfusions were performed in adult beagle dogs with chronically implanted guide tubes, using inulin and ^{42}K contained in a sterile salt solution which was comparable to dog C.S.F. The perfusion fluid was pumped into one lateral ventricle and out of the cisterna magna at a rate of 0·3 ml/min using two equally calibrated modules of a multichannel pump. The experiments were carried out while the animals were conscious and free moving, and pressure alteration within the cerebrospinal fluid system still appeared to be governed by normal physiological variables such as head movement, respiration and blood pressure. The effluent from the perfusion system was collected by means of a fraction collector and the samples were subsequently analysed for inulin, ^{42}K and total potassium. This data, in conjunction with the results of comparable analyses on the inflowing fluid, allowed calculation of bulk flow of C.S.F. from the system and the appropriately corrected rate constants (meq/min) governing the influx and efflux of C.S.F. potassium. All these parameters could thus be assessed on a continuous basis throughout the experiments.

When a stable base line of control values had been established after a period of at least 90 min, the drug under study was administered by either intravenous or intramuscular injection. Of the drugs investigated, the barbiturate anaesthetics, sodium thiopentone and sodium pentobarbitone, when given in doses sufficient to achieve light anaesthesia, had the most striking effects, producing highly significant decreases of up to 40% in both potassium rate constants. Phenytoin and diazepam both produced significant decreases of between 6–14% in efflux and influx rates of C.S.F. potassium in subanaesthetic doses while paraldehyde, even when given in sufficiently high doses to produce light anaesthesia, had no significant effect on potassium fluxes.

These preliminary results would seem to indicate that while the potassium fluxes of

C.S.F. can be reduced by drugs with anaesthetic or anticonvulsant action, these states can be induced by paraldehyde without any significant alteration in C.S.F. potassium.

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